PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A01K 63/00, A61K 39/00, 39/38, 48/00, C12N 5/00, 15/00 — A1

(11) International Publication Number: WO 97/25860

(43) International Publication Date: 24 July 1997 (24.07.97)

(21) International Application Number: PCT/US97/00582

(22) International Filing Date: 13 January 1997 (13.01.97)

(30) Priority Data:
60/010,262 19 January 1996 (19.01.96) US

(71) Applicant *(for all designated States except US)*: ALLEGHENY UNIVERSITY OF THE HEALTH SCIENCES [US/US]; Broad and Vine Streets, Philadelphia, PA 19102-1192 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: HALPERN, Michael, S. [US/US]; 509 Twickenham Road, Glenside, PA 19038 (US). ENGLAND, James, M. [US/US]; 644 West Rosetree Road, Media, PA 19063 (US).

(74) Agent: MONACO, Daniel, A.; Seidel, Gonda, Lavorgna & Monaco, P.C., Suite 1800, Two Penn Center Plaza, Philadelphia, PA 19102 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CELLULAR IMMUNOGENS USEFUL AS CANCER VACCINES

(57) Abstract

A cellular immunogen is provided for immunizing a host against the effects of the product of a target proto-oncogene, where the overexpression of the target proto-oncogene is associated with a malignancy. The cellular immunogen comprises host cells which have been transfected with at least one transgene construct comprising a transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells. The transgene encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene. The transgene may comprise, for example, wild-type or mutant retroviral oncogene DNA cognate to the target proto-oncogene; or wild-type or mutant proto-oncogene DNA of a species different from the host species. The cellular immunogen may be prepared from biopsied host cells, e.g. skin fibroblasts, which are stably or transiently transfected with the transgene construct containing the cognate transgene. The host cells transfected with the cognate transgene construct, are then returned to the body of the host to obtain expression of the cognate transgene in the host.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

"CELLULAR IMMUNOGENS USEFUL AS CANCER VACCINES"

Cross-Reference to Related Application

Priority from U.S. provisional patent application No. 60/010,262, filed January 19, 1996 is claimed.

Field of the Invention

The invention relates to the field of cancer vaccination and immunotherapy.

Background of the Invention

A current goal of cancer research is the identification of host factors that either predispose to tumor formation or serve to enhance tumor growth.

Genes that confer the ability to convert cells to a tumorigenic state are known as **oncogenes**. The transforming ability of a number of retroviruses has been localized in individual viral oncogenes (generally v-*onc*). Cellular oncogenes (generally c-*onc*) present in many species are related to viral oncogenes. It is generally believed that retroviral oncogenes may represent escaped and/or partially metamorphosed cellular genes that are incorporated into the genomes of transmissible, infectious agents, the retroviruses.

Some c-*onc* genes intrinsically lack oncogenic properties, but may be converted by mutation into oncogenes whose transforming activity reflects the acquisition of new properties, or loss of old properties. Amino acid

substitution can convert a cellular proto-oncogene into an oncogene. For example, each of the members of the c-*ras* proto-oncogene family (H-*ras*, N-*ras* and K-*ras*) can give rise to a transforming oncogene by a single base mutation.

Other c-*onc* genes may be functionally indistinguishable from the corresponding v-*onc*, but are oncogenic because they are expressed in much greater amounts or in inappropriate cell types. These oncogenes are activated by events that change their expression, but which leave their coding sequence unaltered. The best characterized example of this type of proto-oncogene is c-*myc*. Changes in *MYC* protein sequence do not appear to be essential for oncogenicity. Overexpression or altered regulation is responsible for the oncogenic phenotype. Activation of c-*myc* appears to stem from insertion of a retroviral genome within or near the c-*myc* gene, or translocation to a new environment. A common feature in the translocated loci is an increase in the level of c-*myc* expression.

Gene amplification provides another mechanism by which oncogene expression may be increased. Many tumor cell lines have visible regions of chromosomal amplification. For example, a 20-fold c-*myc* amplification has been observed in certain human leukemia and lung carcinoma lines. The related oncogene N-*myc* is five to one thousand fold amplified in human neuroblastoma and retinoblastoma. In human acute myeloid leukemia and colon carcinoma lines, the proto-oncogene c-*myb* is amplified five to ten fold. While established cell lines are prone to amplify genes, the presence of known oncogenes in the amplified regions, and the consistent amplification of particular oncogenes in many independent tumors of the same type, strengthens the correlation between increased expression and tumor growth.

Immunity has been successfully induced against tumor formation by inoculation with DNA constructs containing v-*onc* genes, or by inoculation with v-*onc* proteins or peptides. A series of reports describe a form of "homologous" challenge in which an animal test subject is inoculated with either v-*src* oncoprotein or DNA constructs containing the v-*src* gene. Protective immunity was induced against tumor formation by subsequent challenge with v-

src DNA or v-*src*-induced tumor cells. *See*, Kuzumaki *et al.*, *JNCI* (1988), 80:959-962; Wisner *et al.*, *J. Virol.* (1991), 65:7020-7024; Halpern *et al.*, *Virology* (1993), 197:480-484: Taylor *et al.*, *Virology* (1994), 205:569-573; Plachy *et al.*, *Immunogenetics* (1994), 40:257-265. A challenge is said to be "homologous" where reactivity to the product of a targeted gene is induced by immunization with the same gene, the corresponding gene product thereof, or fragment of the gene product. A challenge is "heterologous" where reactivity to the product of a targeted gene is induced by immunization with a different gene, gene product or fragment thereof.

WO 92/14756 (1992) describes synthetic peptides and oncoprotein fragments which are capable of eliciting T cellular immunity, for use in cancer vaccines. The peptides and fragments have a point mutation or translocation as compared to the corresponding fragment of the proto-oncogene. The aim is to induce immunoreactivity against the mutated proto-oncogene, not the wild-type proto-oncogene. WO 92/14756 thus relates to a form of homologous challenge.

EP 119,702 (1984) describes synthetic peptides having an amino acid sequence corresponding to a determinant of an oncoprotein encoded by an oncogenic virus, which determinant is vicinal to an active site of the oncoprotein. The active site is a region of the oncoprotein required for oncoprotein function, e.g., catalysis of phosphorylation. The peptides may be used to immunize hosts to elicit antibodies to the oncoprotein active site. EP 119,702 is thus directed to a form of homologous challenge.

The protein product encoded by a proto-oncogene constitutes a self antigen and, depending on the pattern of its endogenous expression, would be tolerogenic at the level of T cell recognition of the self peptides of this product. Thus, vaccination against cancers which derive from proto-oncogene overexpression is problematic.

Recent attempts have been made to induce immunity *in vitro* or *in vivo* to the product of the HER-2/*neu* proto-oncogene. The proto-oncogene encodes a 185-kDa transmembrane protein. The HER-2/*neu* proto-oncogene is overexpressed in certain cancers, most notably breast cancer. In each report

discussed below, the immunogen selected to induce immunity comprised a purified peptide of the p185$^{HER\text{-}2/neu}$ protein, and not a cellular immunogen.

Disis *et al.*, *Cancer Res.* (1994) 54:16-20 identified several breast cancer patients with antibody immunity and CD4+ helper/inducer T-cell immunity responses to p185$^{HER\text{-}2/neu}$ protein. Antibodies to p185$^{HER\text{-}2/neu}$ were identified in eleven of twenty premenopausal breast cancer patients. It was assumed prior to this work that patients would be immunologically tolerant to HER-2/*neu* as a self-protein and that immunity would be difficult to generate.

Disis *et al.*, *Cancer Res.* (1994) 54:1071-1076 constructed synthetic peptides identical to p185$^{HER\text{-}2/neu}$ protein segments with amino acid motifs similar to the published motif for HLA-A2.1-binding peptides. Out of four peptides synthesized, two were shown to elicit peptide-specific cytotoxic T-lymphocytes by primary *in vitro* immunization in a culture system using peripheral blood lymphocytes from a normal individual homozygous for HLA-A2. Thus, it was concluded that the p185$^{HER\text{-}2/neu}$ proto-oncogene protein contains immunogenic epitopes capable of generating human CD8$^+$ cytotoxic T-lymphocytes.

The cytotoxic T cells elicited in the latter report were not, however, shown to recognize tumor cells, but only targets that bound the synthesized peptides. Other work (Dahl *et al.*, *J. Immunol.* (1996), 157:239-246) has demonstrated that cytotoxic cells may recognize targets that bind peptide but fail to recognize targets that endogenously synthesize peptide. It is thus unclear whether the cytotoxic cells elicited by Disis *et al.* would be capable of recognizing tumor cells. In any event, no protection against tumor growth was demonstrated by Disis *et al.*

Peoples *et al.*, *Proc. Natl. Acad. Sci. USA* (1995), 92:432-436, report the identification of antigenic peptides presented on the surface of ovarian and breast cancer cells by HLA class I molecules and recognized by tumor-specific cytotoxic T lymphocytes. Both HLA-A2-restricted breast and ovarian tumor-specific cytotoxic T lymphocytes recognized shared antigenic peptides.

T cells sensitized against a nine-amino acid sequence of one of the peptides demonstrated significant recognition of HLA-A2 HER2/*neu* tumors.

It remains unclear whether Peoples *et al.* have successfully attacked proto-oncogene-encoded self, as the immunizing peptide which is expressed in the tumor cells contained an isoleucine at position 2, whereas the peptide expressed in normal tissue contains valine residue at this position. Moreover, although stimulation of T cells occurred *in vitro*, this stimulation does not represent a true primary immune response insofar as the starting T cell population represented tumor infiltrating lymphocytes.

The research accounts of Disis *et al.* and Peoples *et al.* required a form of *in vitro* stimulation, either priming as described by Disis *et al.*, or restimulation as described by Peoples *et al.* The *in vitro* protocols of Disis *et al.* and Peoples *et al.* require a mutant cell line to aid in selection of the peptide which will serve to induce reactivity. Non-mutant, peptide antigen-presenting cells have their HLA class I molecules already loaded with endogenous peptides, a phenomenon which precludes exogenous loading from without. The value of the mutant lines is that they lack the TAP genes (encoding the transporters associated with antigen presentation). Class I binding of internally-derived peptides is significantly lowered, and "empty" class I molecules are present on the cell surface and available for binding of exogenously added peptides. This availability of peptide binding sites on membrane-bound class I allows examination of whether a given peptide will (i) even bind to class I, and (ii) function as a target in cytotoxic T cell assays. However, the need for a mutant cell line for deduction of candidate immunizing peptide sequences limits the usefulness of peptide-based immunization schemes.

Fendly *et al.*, *J. Biol. Response Modifiers* (1990), 9:449-455 present an account of a polypeptide-based immunotherapy. Purified polypeptide corresponding to the extracellular domain of the $p185^{HER\text{-}2/neu}$ protein was obtained from a transfected cell line. The purified peptide was employed in the immunization of guinea pigs. The immunized animals developed a cellular immune response, as monitored by delayed-type hypersensitivity. Antisera

derived from immunized animals specifically inhibited the *in vitro* growth of human breast tumor cells overexpressing $p185^{HER\text{-}2/neu}$. There is no indication by Fendly *et al.* of induction of self versus non-self reactivity. It is likely that the guinea pigs were chiefly responding to non-self determinants (as defined in terms of the guinea pig host) on the human polypeptide immunogen.

The use of peptides for immunization is of necessity limited to immunization with a single haplotype. There are approximately thirty HLA types in man. In each case of peptide immunization, one must be careful to select peptides which match the host HLA type. The selected peptide must be immunogenic in the host and be capable of presentation to host immune system cells.

What is needed is an immunization method for immunizing humans and animals against self-encoded proto-oncogenes which are associated with the development of cancer, which dispenses with the need for isolating immunogenic, HLA host-matched peptides for immunization.

Summary of the Invention

It is an object of the invention to induce reactivity to self-determinants of the product of an overexpressed proto-oncogene.

It is an object of the invention to provide for a form of therapy or prophylaxis based upon the capacity to induce immune reactivity to proto--oncogene-encoded self as overexpressed in tumor cells.

It is an object of the invention to provide a cellular immunogen for use in immunization against self proto-oncogene determinants.

It is an object of the invention to provide for a method for vaccinating a host against disease associated with the overexpression of a proto-oncogene.

These and other objects will be apparent from the following disclosure.

A method of vaccinating a host against disease associated with the overexpression of a target proto-oncogene is provided. The method comprises:

(a) excising cells from the host;

(b) transfecting the excised cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene;

(c) returning the excised cells transfected with the transgene construct to the body of the host to obtain expression of the transgene in the host.

According to one principal embodiment of the invention, the transgene comprises wild-type or mutant retroviral oncogene DNA. According to another principal embodiment of the invention, the transgene comprises wild-type or mutant proto-oncogene DNA of a species different from the host species. Where the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA, the mutant DNA is preferably nontransforming. The mutant DNA preferably comprises a deletion mutation in a region of the DNA which is essential for transformation. Preferably, the host cells are transfected with a plurality, most preferably at least five, different transgene constructs, each construct encoding a different deletion mutation.

In one preferred embodiment of the invention, the mutant DNA has at least about 75% homology, more preferably at least about 80% homology, most preferably at least about 90% homology, with the corresponding wild-type oncogene or proto-oncogene DNA.

The invention is further directed to a cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which is associated with a cancer. The cellular

immunogen comprises the host cells which have been transfected with at least one transgene construct, as described above.

The invention is also directed to a method of preparing the cellular immunogen, by (a) excising cells from the host, and (b) transfecting the excised cells with at least one transgene construct, as described above.

The cells transfected with the transgene are preferably rendered non-dividing prior to return to the body of the host.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (*i.e.*, is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence.

The term "cognate" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example but not limitation, in the human genome, the human c-*myc* gene is the cognate gene to the mouse c-*myc* gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode proteins which are functionally equivalent.

By "homology" is meant the degree of sequence similarity between two different amino acid sequences, as that degree of sequence similarity is derived by the FASTA program of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* (1988), 85:2444-2448, the entire disclosure of which is incorporated herein by reference.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

The word "transfection" is meant to have its ordinary meaning, that is, the introduction of foreign DNA into eukaryotic cells.

By "transgene" is meant a foreign gene that is introduced into one or more host cells.

By "transgene construct" is meant DNA containing a transgene and additional regulatory DNA, such as promoter elements, necessary for the expression of the transgene in the host cells.

Description of the Figures

Fig. 1 is a plot of the mean tumor diameter over time following subcutaneous wing web inoculation of 1-day-old line TK (panel A) and line SC (panel B) chickens with 100 μg of tumorigenic plasmids pc*src*527 (—▲—), pVSRC-C1 (—●—) or pMv*src* (--■--). The mean tumor diameter (mm) at a particular time point and for any one group of TK or SC line chickens inoculated was computed as the sum of the diameters of the primary tumors divided by the number of chickens surviving to that point. The ratios at each time point show, for a particular group, the number of chickens bearing palpable tumors to the total number of survivors to that point (standard typeface for pc*src*527, italics for pVSRC-C1, bold typeface for pMV*src*). Error bars (unless obscured by the symbol) indicate standard error.

Fig. 2 is a plot of the growth of challenge (wing web) tumors in test and control line TK chickens under conditions of (i) priming and homologous challenge with plasmid pc*src*527 (panel A: --△--, test; —▲—, control), or (ii) priming and homologous challenge with plasmid pVSRC-C1 (panel B: --○--, test; —●--, control). Test chickens were primed at 1 day posthatch with 100 μg of construct; test and control chickens were challenged at five weeks posthatch with 200 μg of construct. The mean challenge diameter was computed as in Fig. 1. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated (standard typeface for control group, bold typeface for test group). The statistical comparison between the mean challenge tumor diameters of the test versus the control group at a particular time point was made using a two-tailed student's t test, *($p<0.05$), * ($p<0.01$), ***($p<0.001$). The statistical

comparison between the ratios of chickens bearing palpable challenge tumors to total number of survivors of the test versus the control group at a particular time point was made using a chi-squared test; the paired ratios are underlined for only those time points where $p<0.05$. Error bars indicate standard error.

Fig. 3 is a plot of the growth of challenge (wing web) tumors in TK chickens under conditions of (i) priming with plasmid pVSRC-C1 and heterologous challenge with plasmid pc*src*527 (panel A: --△--, test; --▲--, control) or (ii) priming with pc*src*527 and heterologous challenge with pVSRC-C1 (panel B: --○--, test; --●--, control). Test chickens were primed at 1 day posthatch with 100 μg of construct; test and control chickens were challenged at five weeks posthatch with 200 μg of construct. The mean challenge tumor diameter was computed as in Fig. 1. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated (standard typeface for control group, bold typeface for test group). Statistical comparisons were made between test and control groups at a particular time point as described for Fig. 2. [*($p<0.05$), **($p<0.01$), ***($p<0.001$), for the student's t test], and the paired ratios are underlined for only those time points where, in the chi-squared test, $p<0.05$. Error bars indicate standard error.

Detailed Description of the Invention

A vaccination strategy is provided to prevent development of cancers. The vaccination method may be carried out on a subject at risk for a particular cancer, but before the development of the cancer. The practice of the invention may serve for the immunoprevention of prevalent human cancers, such as colon carcinoma, breast carcinoma, and various lymphomas whose progress is accompanied by the overexpression of a cellular proto-oncogene.

The vaccination strategy of the present invention relies on the induction of an immune response that targets tumor cells by virtue of the recognition of the proto-oncogene-specific antigenicity. The aim of the vaccine protocol is to induce reactivity to self-determinants of an overexpressed proto-

oncogene product. The strategy exploits the structural relatedness between the product of the cellular proto-oncogene and that of the product of genes cognate to the target proto-oncogene. The cognate gene may comprise a wild-type or mutant cognate retroviral oncogene or a wild-type or mutant proto-oncogene of a species different from the host species. The starting point of the vaccine strategy is the high degree of primary sequence homology that exists between the protein product of a targeted proto-oncogene and that of its cognate retroviral oncogene, or between the proto-oncogene product and the product of a cognate proto-oncogene from a different species. However, in contrast to other proposed vaccine strategies, the present invention is not based on the immune recognition of a determinant defined by a cancer specific mutation.

For those tumors showing proto-oncogene overexpression, this sequence homology permits application of the following strategy, which can be employed either prophylactically or therapeutically under conditions of cell-surface expression, or other forms of adjuvanicity, as chosen to enhance immunogenicity: (a) immunization of host biopsied cells with a DNA construct comprising a transgene cognate to the target proto-oncogene, which transgene encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene; (b) return of the transfected cells to the body of the host to obtain expression of the transgene in the host, and thus immunity against the proto-oncogene product. The invention relies on the targeting of a self-determinant found on an overexpressed or overabundant proto-oncogene-encoded product. The foreign peptide elements of the immunizing oncogene product will trigger peripheral lymphocytes exhibiting a weak cross reactivity for the self peptides of the targeted proto-oncogene product. Although such self peptides would be present in normal cells expressing the proto-oncogene, targeting of the tumor cells is favored in view of their overexpression of the proto-oncogene.

The immune strategy exploits the antigenicity of two alternative types of determinants: (1) tumor-associated antigenic determinant(s) induced as a consequence of the activity of the oncogene product, e.g., an enzymatic

modification of a cellular protein effected by the oncogene product, or (2) tumor associated antigenic determinant(s) intrinsic to the oncogene-encoded product itself. The difficulty in exploiting the first alternative by traditional means, i.e., antigen purification, is that at present little or no systematic information exists bearing on the properties of an antigen that, though oncogene-induced, is not oncogene-encoded. This situation makes purification of any such antigen problematic. However, this problem is obviated from the outset by the present invention which utilizes biopsied cells which, as transfected in culture by the cognate retroviral oncogene, would express the relevant antigenicity.

In terms of exploiting the second alternative, that of an antigenicity intrinsic to the proto-oncogene product, a relevant consideration is that the protocol of immunization according to the present invention primes the host to determinants of the oncogene product itself. A consequence of this immunization is induction of T-cell reactivity to the divergent, i.e foreign, peptide determinants of the retroviral oncogene product, i.e., those peptide determinants that show sequence differences with the positionally homologous determinants of the cellular proto-oncogene product. The induction of this reactivity does not in itself have vaccine potential, since the foreign determinants specific to the retroviral oncogene product are normally absent from the cellular proto-oncogene product. Nevertheless, the foreign peptide elements, notably those that differ by only a single amino acid from the positionally homologous self peptides, trigger peripheral T-lymphocytes exhibiting a weak cross-reactivity for the self peptides. Although such self peptides are present in normal cells expressing the proto-oncogene, targeting of the tumor cells is favored in view of their overexpression of the proto-oncogene.

It is possible that many tumor-associated and overexpressed proto-oncogenes might possess mutations. In some cases, overexpression may very well arise as a direct consequence of one or more of the mutations. However, the present vaccination method does not have as its object the deliberate targeting of non-self determinants generated by proto-oncogene mutations. Unlike prior vaccination methods designed to target such mutation-

driven non-self determinants, it is the aim of the present invention to induce reactivity for self-determinants in the overexpressed product of tumor associated and overexpressed proto-oncogenes.

Prior efforts attempting to elicit reactivity to proto-oncogene self determinants have relied on *in vitro* protocols utilizing mutant cell lines to identify individual self peptide immunogens (Disis *et al.*, *Cancer Res.* (1994) 54:1071-1076; Peoples *et al.*, *Proc. Natl. Acad. Sci USA* (1995), 92:432-436). According to the present invention, the host immune system is presented with the full array of naturally-derived class I binding peptides. The vaccine strategy of the present invention obviates the need for any *a priori* assessment of the immunogenicity of individual peptides.

While the cellular immunogens of the invention display self peptides, non-self peptides would also be presented which may serve as more effective tolerance breakers. The value of a non-self, but closely related to self, peptide is that it may more readily activate those T cells that have both a weak cross reactivity for the cognate self peptide and an activation threshold (determined by the tightness of binding to the T cell receptor) too high to be triggered by the self peptide. Moreover, cognate non-self is inductive of a good immune response, simply because it does in fact constitute nonself. The non-self immune response is expected to predispose the induction of the inevitably weaker response to the self determinants on the same protein product, since the resultant cytokine release provides local help to initiate the weaker anti-self response.

As hereinafter exemplified in a model of *src*-oncogene-based tumor formation, immunization with cells transfected with a transgene construct expressing the v-*src* oncogene product induces reactivity to the product of the c-*src* proto-oncogene, thereby conferring protection against the growth of tumors displaying overexpression of the c-*src* proto-oncogene.

Target Proto-Oncogenes

According to the present invention, patients with a family history of a cancer characterized by the overexpression of a particular proto-oncogene are selected for immunization. Alternatively, patients whose tumors can be shown to overexpress the proto-oncogene are selected. Overexpression of a proto-oncogene may derive from an increase over a basal level of transcription. Overexpression may also derive from gene amplification, that is, an increase in gene copy number, coupled with a basal or elevated level of transcription. Proto-oncogene overexpression may be assayed by conventional probing techniques, such as described in *Molecular Cloning: A Laboratory Manual* J. Sambrook *et al.*, eds., Cold Spring Harbor Laboratory Press, 2nd ed. 1989. The level of target proto-oncogene expression may be determined by probing total cellular RNA from patient cells with a complementary probe for the relevant mRNA. Total RNA from the patient cells is fractionated in a glyoxal/agarose gel, transferred to nylon and hybridized to an appropriately labelled nucleic acid probe for the target mRNA. The number of relevant mRNA transcripts found in the patient cells is compared to that found in cells taken from the same tissue of a normal control subject.

As an alternative to measuring mRNA transcripts, the expression level of a target proto-oncogene may be assessed by assaying the amount of encoded protein which is formed. Western blotting is a standard protocol in routine use for the determination of protein levels. See *Molecular Cloning, supra*, Chapter 18, incorporated herein by reference. Accordingly, a cell lysate or other cell fraction containing protein is electrophoresed on a polyacrylamide gel, followed by protein transfer to nitrocellulose, and probing of the gel with an antibody specific for the protein in question. The probe step permits resolution of the desired protein from all other proteins in the starting mixture. The bound antibody may be prelabeled, *e.g.*, by a radioisotope such as ^{125}I, so as to permit its detection on the gel. Alternatively, a secondary reagent (usually an anti-immunoglobin or protein A) may be radiolabeled or covalently coupled

to an enzyme such as horseradish peroxidase or alkaline phosphatase. The strength of the signal is proportional to the amount of the target protein. The strength of the signal is compared with the signal from a sample analyzed in the same manner, but taken from normal as opposed to tumor tissue.

A description of the methodology and use of Western blotting to determine the levels of the c-*src*-encoded protein $pp60^{c\text{-}src}$ in adenomatous polyps (colonic epithelia) is provided by Cartwright *et al.*, *Proc. Natl. Acad. Sci. USA* (1990), 87:558-562, the entire disclosure of which is incorporated herein by reference.

An at least about eight-fold increase in that gene's expression in the patient cells compared to expression in normal control cells from the same tissue would indicate candidacy for vaccination.

Table 1 includes a partial list of representative proto-oncogenes, the overexpression of which has been associated with one or more malignancies. Each listed proto-oncogene is a target proto-oncogene according to the present invention. The corresponding oncogene, of which the target proto-oncogene is the normal cellular homolog, is also identified. This list of target proto-oncogenes is intended to be representative, and not a complete list.

Table 1

Representative List of Target Proto-Oncogenes

Proto-Oncogene	Tumor	Comments/References
AKT-2	ovarian	v-*Akt* is the oncogene of the AKT8 virus, which induces lymphomas in mice. 1. Bellacosa *et al.*, (1995) *Int. J. Cancer* 64(4):280-5: Southern-blot analysis has shown AKT-2 amplification in 12.1% of ovarian carcinomas, while Northern bot analysis has

			revealed overexpression of AKT-2 in 3 of 25 fresh ovarian carcinomas which were negative for AKT-2 amplification.
			2. Cheng *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 89(19): 9267-71): Amplification of AKT-2 has been detected in 10% of pancreatic carcinomas.
	AKT-2	pancreatic	Cheng *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 93(8):3636-41: Amplification of AKT-2 has been detected in 10% of pancreatic carcinomas.
	c-*erb*B-2	bladder	c-*Erb*B-2 is also known as HER2/*neu*. V-*erb*B is the oncogene of the avian erythroblastosis virus.
			1. Underwood *et al.*, (1995) *Cancer Res.* 55(11):2422-30: Protein overexpression was observed in 45% of patients with non-recurrent disease and 50% of patients with recurrent disease; 9% of bladder tumors analyzed shoed gene amplification.
			2. Coombs *et al.*, (1993) *Pathology* 169(1):35-42: c-*Erb*B-2 gene amplification was observed in 14% of bladder tumors analyzed.
			3. Gardiner *et al.*, (1992) *Urolog. Res.* 20(2):17-20: Nineteen percent of primary transitional cell bladder carcinomas showed c-*erb*B-2 gene amplification.
	c-*erb*B-2	breast	1. Molina *et al.*, (1966) *Anticancer Research* 16(4B):2295-300: Abnormal c-*erb*B-2 levels were found in 9.2% of patients with locoregional breast

carcinoma, and in 45.4% of patients with advanced disease. 2. DePotter *et al.*, (1995) *Virchows Arch.* 426(2):107-15: Overexpression of the oncoprotein is observed in about 20% of invasive duct cell carcinomas of the breast. 3. Bandyopadhyay *et al.*, (1994) *Acta Oncol.* 33(5):493-8: 35.4% of breast tumors showed c-*erb*B-2 overexpression; 17.4% showed gene amplification. 4. Fontana *et al.*, (1994) *Anticancer Res.* 14(5B):2099-104: 26% of samples showed c-*erb*B-2 amplification. 5. Press *et al.*, (1993) *Cancer Research* 53(20):4960-70: Amplified overexpression was identified in 38% of primary breast cancers. 6. Berns *et al.*, (1992) *Cancer Res.* 52(5):1107-13: 23% of primary breast cancer tissues exhibited amplification. 7. Delvenne *et al.*, (1992) *Eur. J. of Cancer* 28(2-3):700-5: c-*erb*B-2 mRNA was overexpressed in 34% of breast tumor samples. 8. Inglehart, (1990) *Cancer Res.* 50(20):6701-7: Two to thirty-two-fold gene amplification was found in multiple stages of tumor progression. 9. Slamon *et al.*, (1989) *Science* 244:707-12: A 28% incidence of amplification of c-*erb*B-2 was found in 189 primary breast cancers. 10. Kraus *et al.*, (1987) *EMBO J.* 6(3):605-10: Eight cell lines demonstrated c-*erb*B-2 mRNA levels ranging from 4 to 128-fold overexpression. 60% of all tumors analyzed showed elevated levels of c-*erb*B-2 mRNA.

c-*erb*B-2	lung	1. Osaki *et al.*, (1995) *Chest* 108(1):157-62: Lung tissue overexpression of c-*erb*B-2 was discovered in 42.5% of samples. 2. Lorenz *et al.*, (1994) *Clin. Invest.* 72(2):156-63: A 64-fold increase in the amount of c-*erb*B-2 mRNA was observed; 33% of lung tumors showed overexpression of c-*erb*B-2.
c-*erb*B-2	ovarian	1. Katsaros *et al.*, (1995) *Anticancer Res.* 15(4):1501-10: Abnormally high expression of c-*erb*B-2 was found in 31% of tumor samples. 2. Felip *et al.*, (1995) *Cancer* 75(8):2147-52: 21.7% of ovarian tumors showed overexpression of c-*erb*B-2. 3. Fan *et al.*, (1994) *Chin. Med. J.* 107(8):589-93: c-*erb*B-2 amplification was found in 30.8% (8 of 26) of human ovarian cancers. 4. vanDam *et al.*, (1994) *J. of Clin. Path.* 47(10):914-9: 24% of ovarian tumors showed c-*erb*B-2 overexpression. 5. Csokay *et al.*, (1993) *Eur. J. of Surg. Oncology* 19(6):593-9: c-*erb*B-2 amplification was found in 34% of fresh ovarian tumor samples. 6. McKenzie *et al.*, (1993) *Cancer* 71(12):3942-5: 30% of ovarian tumor samples indicated c-*erb*B-2 overexpression. 7. Hung *et al.*, (1992) *Cancer Letters* 61(2):95-103: A 100-fold c-*erb*B-2 overexpression was discovered in one human cell line. Two to four-fold amplification was also discovered.
MDM-2	leukemia	*MDM*-2 is the murine double minute-2 oncogene. 1. Bueso-Ramos *et al.*, (1993) *Blood* 82(9):2617-

		23: 53% of cases showed overexpression of *MDM*-2 mRNA. The level of *MDM*-2 mRNA overexpression in some cases of leukemias was comparable to that observed in some sarcomas, which demonstrate more than 50-fold *MDM*-2 gene amplification. No evidence of gene amplification was observed. 2. Watanabe *et al.*, (1994) *Blood* 84(9):3158-65: 28% of patients with B-cell chronic lymphocytic leukemia or non-Hodgkin's lymphoma had 10-fold higher levels of *MDM*-2 gene expression. *MDM*-2 overexpression was found more frequently in patients at advanced clinical stages.
c-*myb*	colon	V-*myb* is the oncogene of the avian myeloblastoma virus. 1. Ramsay *et al.*, (1992) *Cell Growth and Diff.* 3(10):723-30: c-*myb* levels were always higher in colon cancer samples than normal tissue. 2. Alitalo *et al.*, (1984) *Proc. Natl. Acad. Sci.* 81(14):4534-8: c-*myb* levels were always higher in colon cancer samples than normal tissue.
c-*myc*	breast	V-*myc* is the oncogene of the avian myelocytoma virus. 1. Lonn *et al.*, (1995) *Cancer* 75(11):2681-7: Amplification of c-*myb* occurs in 16% of patients with breast cancer. 2. Hehir *et al.*, (1993) *J. of Surg. Oncology* 54(4):207-9: c-*myc* overexpression was found in 60% of breast carcinoma samples. 3. Kreipe *et al.*, (1993) *Cancer Research* 53(8):1956-61: Amplification of

		c-*myc* was found in 52.6% of samples that displayed a Ki-S1 labelling index exceeding 30%. 4. Watson *et al.*, (1993) *J. Nat. Cancer Inst.* 85(11):902-7: Amplification of c-*myc* occurs in up to 20 - 30% of breast cancers. 5. Berns *et al.*, (1992) *Cancer Research* 52(5):1107-13: Amplification was found in 20% of primary breast cancer patients; the range was 3-14 gene copies. 6. Watanabe *et al.*, (1992) *Cancer Research* 52(19):5178-82: Expression of c-*myc* was increased by 10-fold.
c-*myc*	gastric/ colorectal	1. Rigas, (1990) *Clin. Gastroent.* 12(5):494-9: Overexpression of c-*myc* is found in 80 of colon cancers. 2. Erisman *et al.*, (1988) *Oncogene* 2(4):367-78: Adenocarcinoma cell lines express 5-10-fold elevated levels of c-*myc* mRNA. Eight to thirty-seven-fold higher levels of c-*myc* protein was found in tumor cell lines compared to normal cells. 3. Sikora *et al.*, (1987) *Cancer* 59(7):1289-95: Up to 32-fold overexpression of c-*myc* mRNA was observed in 12 to 15 tumors. 4. Tsuboi *et al.*, (1987) *Biochem. and Biophys. Res. Comm.* 146(2):705-10: Gastric Cancer: A 2-3-fold overexpression was observed in gastric cancer. A 2-10-fold overexpression was observed in colorectal cancer.
c-*myc*	lung	1. Lorenz *et al.*, (1994) *Clin. Invest.* 72(2):156-63: A 57-fold increase in c-myc mRNA levels was observed. 23% of samples indicated strong

		expression of c-myc. 2. Kato *et al.*, (1993) *Jap. J. of Cancer Res.* 84(4):355-9: Liver tissue metastases from human small cell lung carcinoma revealed 30-fold amplification of c-*myc*.
c-*myc*	naso-pharn-geal	Porter *et al.*, (1994) *Acta Oto-Laryng.* 114(1): 1105-9: 22% of samples showed intense staining for c-*myc*.
c-*myc*	ovarian	1. Bian *et al.*, (1995) *Chin. J. of Ob. Gyn.* 30(7):406-9: 50% of samples showed amplification of c-*myc*. 2. Katsaros *et al.*, (1995) *Anticancer Res.* 15(4):1501-10: 26% of samples exhibited c-*myc* amplification. 3. van Dam *et al.*, (1994) *J. Clin. Path.* 47(10):914-9: Overexpression of c-*myc* was found in 35% of ovarian carcinomas. 4. Xin *et al.*, (1993) *Chin. J. of Ob. Gyn.* 28(7):405-7: 54.5% of samples showed amplification of c-*myc*. 5. Tashiro *et al.*, (1992) *Int. J. of Cancer* 50(5):828-33: Overexpression was found in 63.5% of all serous adenocarcinoma tissues and 37.3% of all ovarian carcinoma tissues. Significant overexpression of c-*myc* was observed at Stage III compared with other stages.
c-*myc*	prostate	Nag *et al.*, (1989) *Prostate* 15(2):115-22: A 10-fold amplification of c-*myc* was observed. Fifty-fold higher levels of mRNA transcripts of c-*myc* were found.

c-*ras*	lung	*Ras* oncogenes were first recognized as the transforming genes of Harvey and Kirsten murine sarcoma viruses. Lorenz *et al.*, (1994) *Clin. Invest.* 72(2):156-63: a 13-fold increase in overexpression of c-Ki-*ras* was observed. 18% of tumors displayed strong overexpression of c-Ki-*ras*.
c-*ras*	ovarian	1. Katsaros *et al.*, (1995) *Anticancer Res.* 15(4):1501-10: Higher levels of *ras* protein than in normal or benign ovarian tumors were found in 45% of tumor samples. 2. vanDam *et al.*, (1994) *J. of Clin. Path.* 47(10):914-9: 20% of ovarian tumors exhibited c-*ras* overexpression. The levels of expression of c-*ras* were much higher in tumors of patients with recurrent or persistent disease after chemotherapy, than in the tumors of patients at initial presentation.
c-*src*	breast	V-*src* is the oncogene of the Rous sarcoma virus, which induces sarcomas in chickens. Muthuswamy *et al.*, (1994) *Mol. and Cell. Biol* 14(1):735-43: c-*erb*B-2-induced mammary tumors possessed 6-8-fold higher c-*src* kinase activity than adjacent epithelium.
c-*src*	colon/ colorectal	1. Cartwright *et al.*, (1994) *J. of Clin. Invest.* 93(2):509-15: c-*src* activity is 6-10-fold higher in mildly dysplastic ulcerative colitis (a chromic inflammatory disease of the colon with a high on incidence of colon cancer) than in non-dysplastic

		epithelia. This data suggests that activation of c-*src* is an early event in the genesis of UC colon cancer. 2. Talamonti *et al.*, (1993) *J. of Clin. Invest.* 91(1):53-60: High level of c-*src* activity from colorectal cancer is found in liver metastases. 3. Termuhlen *et al.*, (1993) *J. of Surg. Res.* 54(4):293-8: Colon carcinoma metastases to the liver had significantly increased activity of c-*src* with an average 2.2-fold increase. Extrahepatic colorectal metastases demonstrated an average 12.7-fold increase in c-*src* activity over normal mucosa.
	c-*yes* colon	V-*yes* is the oncogene of two avian sarcoma viruses, Esh sarcoma virus and Y73. 1. Pena *et al.*, (1995) *Gastroent.* 108(1):117-24: Twelve to fourteen-fold higher expression of c-*yes* was found in colonic transforming oncogene adenomas compared to normal mucosa. Activity of c-*yes* was elevated in adenomas that are at greatest risk for developing cancer. 2. Park *et al.*, (1993) *Oncogene* 8(10):2627-35: A ten to 20-fold higher than normal activity of c-*yes* was observed in 3 out of 5 colon carcinoma cell lines. A 5-fold higher than normal activity was found in 10 out of 21 primary colon cancers, compared to normal colonic cells.

Selection of Cognate Transgene for Preparation of Cellular Immunogen

According to the present invention, a transgene construct is engineered comprising a transgene which is cognate to the target proto-oncogene (hereinafter "cognate transgene" or "CTG"). The transgene is selected such that it encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene. The transgene should be expressed to very high levels in the transfectants. Thus, the construct should contain a strong promoter.

The product encoded by the cognate gene must have a high degree of sequence homology with the product of the target proto-oncogene, but also must display some amino acid differences with the target proto-oncogene product. Thus, there must be a subset of one or more amino acid differences between the target proto-oncogene and its cognate in order to provide immunogenic stimulus. Two classes of genes that satisfy these criteria are retroviral oncogenes and xenogenic proto-oncogenes. The word "xenogenic" is intended to have its normal biological meaning, that is, a property or characteristic referring or relating to a different species. Thus, a xenogenic proto-oncogene is meant to include the a homologous proto-oncogene of a species other than the host organism species. It may be appreciated that in the case of a target proto-oncogene, e.g. MDM2, for which no retroviral homolog is yet known, a xenogenic homologue is advantageously utilized as the source of the DNA for the cognate transgene.

In principle, a more effective immunogenic stimulus would depend on the particular sequence, and not on the distinction between a retroviral oncogene and a xenogenic proto-oncogene in terms of their relative transforming capacity. Thus, in certain cases, a retroviral oncogene may be better at providing a tolerance-breaking immunogenic stimulus, and in other cases, a xenogenic proto-oncogene may be more effective.

The retroviral oncogene or xenogenic proto-oncogene DNA forming the CTG may comprise the wild type oncogene or proto-oncogene DNA. More preferably, a mutant DNA is utilized, which is engineered so as

to be non-transforming in the host. The DNA is mutated to include one or more nucleotide insertions, deletions or substitutions which will encode an oncogene product which is nontransforming in the host, but retains the requisite degree of sequence homology with respect to the target proto-oncogene. A cognate transgene deletion mutant (hereinafter "dCTG") is preferred.

A protein sequence is generally considered "cognate" with respect to the target proto-oncogene-encoded protein if it is evolutionarily and functionally related between species. A more precise view of cognation is based upon the following sequence comparison carried out utilizing the FASTA program of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* (1988), 85:2444-2448, the entire disclosure of which is incorporated herein by reference. Cognation is attained upon satisfying two criteria imposed by FASTA; (i) alignment of segments corresponding to at least 75% of the target proto-oncogene's encoded amino acid sequence; (ii) at least 80% amino acid identity within the aligned sequences. The segments of the target proto-oncogene protein sequence and protein test sequence satisfying the two criteria are referred to as "homology regions". Accordingly, at least 75% of the target proto-oncogene protein sequence is alignable with the test sequence. The alignable segments or homology regions may, however, represent less than 75% of the total test polypeptide chain for the case of test sequences that may significantly exceed the target proto-oncogene protein in length.

One skilled in the art, armed with the FASTA program, may survey existing sequence data bases (either protein sequences or DNA sequences, insofar as the amino acid sequence is determined by FASTA for all reading frames) for test sequences which are cognate with respect to the target proto-oncogene. At the same time, one can isolate and then sequence what are very likely to be cognate test sequences (*e.g.* feline MDM-2, as likely to be cognate to human MDM-2) and use FASTA to verify the presumed cognation, according to the criteria set above. One may obtain the sequences of presumptive cognate proto-oncogenes from a large number of mammalian

sequences and screen these sequences with FASTA according to the aforesaid formulation of cognation.

Because the product encoded by a CTG differs at a small number of amino acid positions from the product encoded by the target proto-oncogene, an immunogenic stimulus is provided that (i) is directed against the foreign protein and (ii) with a lower probability, induce an anti-self response. The CTG is selected such that the gene product will yield the greatest immunogenic stimulus to induce anti-self reactivity. Provided that overall sequence homology (preferably greater than about 75%) is maintained, the presence of scattered amino acid differences is desired, since any one residue would likely have a relatively low probability of inducing self-reactivity. Moreover, the greatest number of residue differences would be advantageous, consistent with maintaining the requisite degree of general sequence homology.

The selection of amino acid modifications for the CTG may be facilitated by resort to available computer-based models used to identify immunogenic peptide fragments of polypeptides. These models could be employed to select CTGs which would possess the maximum number of immunogenic peptides for a given HLA haplotype.

Screening Procedure for CTG Selection

Notwithstanding the availability of computer-based algorithms which have some predictive value, it is desirable to design CTGs with resort to a screening procedure based on an actual experimental assay that can be HLA-haplotype specific. Accordingly, cells are biopsied from a normal volunteer of particular haplotype. The cells are transfected with a CTG construct, preferably a dCTG construct, satisfying the criteria set for cognition. More preferably, the cells are transfected with multiple dCTGs, preferably at least five dCTGs, satisfying the criteria for cognition. The at least five dCTGs are selected to display amino acid differences that essentially extend throughout the polypeptide chains of the encoded sequences. The transfected cells are then used to immunize the volunteer in accordance with the immunization method of the

present invention. After immunization, the human subject is tested in a standard delayed hypersensitivity (DH) reaction with 10^4-10^6 irradiated, autologous fibroblasts, as transfected with the same dCTG (or series of dCTGs) as used for the immunizing preparation. A positive DH reaction (induration) would verify the induction of reactivity. The induction of reactivity in this assay is readily demonstrable because of the priming to the non-self determinants on the dCTG-encoded protein and the readout in the DH reaction of the same nonself determinants. Once DH reactivity is demonstrated in a DH reaction that directly tests the antigenicity of the non-self determinants encoded by the dCTG (*i.e.*, priming with a non-self construct, DH testing with the same non-self construct), the subject can be then tested in a DH reaction based on testing with the autologous cells transfected with a dCTG derived from the human proto-oncogene itself (*i.e.*, priming with a non-self construct, testing with the human self construct). Testing of a battery of human volunteers will lead to a catalogue of HLA-matched dCTGs, such that, for individuals of the same HLA haplotype, the use of the particular dCTG would be inductive of reactivity to proto-oncogene-encoded self. Different CTGs may thus be tested so as to correlate maximal secondary stimulation with a particular HLA haplotype.

At the same time, this procedure may be used with patients undergoing tumor resection (if post-operative immuno-suppressive protocols are not mandatory), such that prior to resection, a course of immunization would have been initiated, the endpoint of which would represent the development of a DH reaction.

Any given amino acid difference between the CTG-encoded product and the proto-oncogene-encoded product has a low probability of being a "tolerance-breaker". Thus, it is preferable to transfect the host cells with a mixture of multiple different CTGs, preferably dCTGs. The number of different dCTGs is preferably five or more. Moreover, it is preferred that, among themselves, the multiple dCTGs show amino acid differences that essentially extend throughout the polypeptide chains of the encoded sequences. The dCTGs would be selected to maximize amino acid differences and, at the

same time, make sure that differences are found all along the polypeptide chain. It would thus not be preferable to select a battery of deletions all from within the same domain of the polypeptide chain.

According to a protocol which utilizes 10^7 irradiated cells for immunization containing five separate dCTGs, five groups of 2 X 10^6 cells are included in one inoculate, each group of 2 X 10^6 having been transfected with a separate dCTG from the total set of five CTGs that are cognate to a particular proto-oncogene.

Selection of Non-Transforming Cognate Transgenes

Non-transforming cognate transgene variants are most advantageously derived via deletion of a sequence essential for transformation. Unlike point mutations which are potentially reversible due to back mutations, deletion mutations are irreversible. Furthermore, deletion mutations do not possess the inherent disadvantage attaching to point mutations, namely, even though the requirement for generation of an acceptable cognate transgene is for a qualitative difference with the wild type, i.e., non-transforming versus transforming, any given point mutation may be neutral or else quantitative in its effect, that is, the mutation may reduce but not totally eliminate transformability. Thus, according to a preferred embodiment of the invention, a deletion is created in a region of the cognate transgene which encodes an amino acid sequence required for transformation. Consonant with non-transformability, the smallest deletion possible so as to leave intact the bulk of the antigenicity of the transgene product is selected.

The engineering of a cognate transgene deletion mutant that satisfies these criteria is facilitated by reports of structure-function relationship in oncogene-encoded proteins. Such reports serve to identify regions of oncoproteins that are essential for transformation, as opposed to regions which are either neutral or serve merely to modulate transformability. Although such reports are usually based on *in vitro* transformation assays, and are therefore independent of immune effects, these studies can be exploited to aid in the

construction of non-transforming dCTGs for use in the practice of the present invention.

The deletion mutant is engineered to include at least a part of the region identified as critical for transformation. In those cases where essential amino acids have been identified, the deletion will span these residues. The engineering of any desired deletion can be readily accomplished by polymerase chain reaction (PCR) according to conventional PCR techniques, based upon the known nucleotide sequence of the unmutated cognate transgene.

The following describes a representative protocol for deriving a non-transforming dCTG of the smallest possible deletion, for use in the practice of the present invention. A test dCTG, engineered on the basis of known or ascertained transformation-specific domains, and driven by the strongest possible promoter, is used to transfect murine 3T3 cells. A sister culture of 3T3 cells is also transfected, with non-deleted CTG. Each CTG or dCTG cell culture is inoculated into nude mice, in the absence of any treatment to render the cells non-dividing. Those dCTGs which do not yield tumors in the mice even after prolonged observation are then utilized as transgenes for the biopsied human cells which, upon transfection with the transgene, will serve as a cellular vaccine according to the practice of the present invention. The dCTGs are selected with the smallest deletion mutant consonant with non-transformability.

Some CTGs representing xenogenic proto-oncogenes may not be tumorigenic in the 3T3/nude mouse assay. For any such non-transforming CTG, it is not essential to generate a dCTG. However, even given non-tumorigenicity in nude mice, it may be desirable to opt for generation of a deletion mutant when the transgene is based upon a xenogenic proto-oncogene. In such cases, the deletion would be engineered so as to remove the homologous region to that deleted in the particular dCTG that corresponds to the deletion in the corresponding retroviral oncogene dCTG.

Even though the transgene construct may comprise mutant oncogene or proto-oncogene DNA which is nontransforming, it is nevertheless preferable, as a safety measure, to treat the transfected cells to render them non-

dividing before inoculation back into the host. The cells are irradiated with a radiation dosage sufficient to render them non-dividing.

Oncogenicity Assay of Cognate Transgenes

As a further safety measure, the oncogenicity of a given dCTG is preferably thoroughly tested prior to infection of the human host cells which are used as cellular immunogens according to the practice of the present invention. For example, an oncogenicity testing regimen may take the form of three separate assays: (i) dCTG transfection of NIH 3T3 cells, followed by inoculation into nude mice; (ii) dCTG transfection of human fibroblasts, followed by inoculation into nude mice; and (iii) dCTG transfection of human fibroblasts, followed by an *in vitro* test of anchorage-dependent growth. In principle, all three should be negative to validate the use of any given dCTG in the vaccination method of the present invention.

According to the oncogenicity assay (i), after stable transfection of NIH 3T3 cells with the test dCTG, the transfectants are inoculated into nude mice. Tumorigenicity of the transfectants in the mice is then evaluated according to standard protocols.

According to oncogenicity assay (ii), human fibroblasts are transfected with the test dCTG as proposed in the above human immunization protocol. After stable dCTG transfection of human fibroblasts, however, rather than carrying out X-irradiation of the transfectants to render them non-dividing, followed by inoculation of the irradiated transfectants back into the human host, the transfectants are directly inoculated into nude mice as a direct test of tumorigenicity. Given the greater susceptibility of murine 3T3 cells to oncogenic transformation, *vis a vis* primary human or murine transfectants fibroblasts, assay (ii) is probably much less sensitive than assay (i), but does have the advantage of offering a direct test of dCTG oncogenicity in human cells.

According to oncogenicity assay (iii), non-irradiated dCTG-transfected human fibroblasts are assayed for anchorage-dependent growth, *i.e.*

colony formation in soft agar, as a test of dCTG transforming potential in human cells. Anchorage independence, as defined by the ability of cells to grow when suspended in semisolid medium, is a common phenotype acquired by human tumor cells, particularly those tumor cells of mesenchymal origin, such as fibrosarcomas. While assay (iii) has no *in vivo* readout, it offers an independent test of the critical issue of dCTG oncogenicity in human cells.

The oncogenicity assays are performed according to published protocols. Assay (i), comprising dCTG transfection of NIH 3T3 cells followed by inoculation into nude mice, may be performed according to the protocol of Stevens *et al.*, *Proc. Natl. Acad. Sci. USA* (1988), 85:3875-3879, including DNA transfection by the calcium phosphate coprecipitation method of Manohaven *et al.*, *Carcinogenesis* (1985), 6:1295-1301. Accordingly, NIH 3T3 cells (7.5 X 10^5 cells per 100-mm dish) are exposed to a calcium phosphate-DNA coprecipitate (40 μg of genomic DNA plus 3 μg of pSV2neo per dish) for 4 hours. Two days later, each dish is trypsinized and reseeded into a 175-cm^2 flask. For the next 10 days, cultures are selected in G418 (400 μg/ml), and the flasks are then trypsinized and cells are replated in the same flask to disperse the G418-resistant colonies into a diffuse lawn of cells. Two days later, the cells are harvested and washed with serum-free medium prior to injection. One injection of 5 X 10^6 cells into the right flank and one injection of 1 X 10^7 cells into the left flank, each in a volume of 200 μl, are done on each nude mouse. Injection sites are monitored at 3- or 4-day intervals for 100 days. The sites are scored for the number of tumors induced per injection site.

Oncogenicity assay (ii), whereby dCTG transfection of human fibroblasts followed by inoculation into nude mice, is carried out in the same manner as assay (i) except that for assay (ii) the human fibroblast transfectants are substituted for the murine 3T3 transfectants.

Assay (iii), involves a test of the *in vitro* anchorage-dependent growth of dCTG-transfected human fibroblasts. The assay is carried out as described in Stevens *et al.*, *J. Cancer Res. and Clin. Oncol.* 1989, 115:118-128. 1 x 10^5 cells are seeded per 60-mm dish into 0.33% Noble agar over a

6-ml 0.5% agar base layer in Hams F10 supplemented with 6% fetal bovine serum. A portion of the agar suspension is diluted with Hams F10 plus 6% fetal calf serum to 200 cells/5 ml to determine the cloning efficiency of these cells when seeded into plastic 60-mm dishes. Agar dishes are fed with 1 ml Hams F10 supplemented with 6% fetal bovine serum on the 1st and 15th day after seeding. Four weeks after seeding, all agar colonies >75 μm in diameter are counted and the colony counts are normalized to the plating efficiencies which aliquots of the initially seeded cells showed on plastic. This comparison, or normalization, of the agar colony counts to the plastic dish colony counts is useful in identifying and correcting for any mechanical artifacts which might result from the seeding into agar of dead cells that had persisted from the initial transfection treatment or from heat-induced cell death, which might have occurred while suspending cells in molten agar during the process of seeding the agar dishes.

The following is a partial list of various deletions which, based upon published accounts of experiments with human or animal cells, are believed to render the identified CTG non-tumorigenic.

Table 2

Deletion Mutations Rendering Indicated Gene Non-Transforming

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
Akt-2 (c-akt) (mouse)	M95936; SEQ ID NO:3 (*Mus musculus* serine/threonine kinase)	480	148-234	Bellacosa *et al.*, *Science* (1991), 254:274-278; Bellacosa *et al.*, *Oncogene* (1993), 8(3):745-54.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
c-*neu* (c-erbB-2) (rat)	M11730; SEQ ID NO:4 (human tyrosine kinase-type receptor (HER2) gene	1255	1-731	Bargmann *et al.*, *EMBO* (1988), 7(7):2043-52; Bernards *et al.*, *Proc. Natl. Acad. Sci. USA* (1987), 84(19):6854-8.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
mdm-2 (human)	U33199; SEQ ID NO:5 (human mdm2-A mRNA); U33200; SEQ ID NO:6 (human mdm2-B mRNA); U33201; SEQ ID NO:7 (human mdm2-C mRNA); U33202; SEQ ID NO:8 (human mdm2-D mRNA); U33203;	489	9-155	Dubs-Poterszman, *Oncogene* (1995), 11(11):2445-50.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
c-*myb* (human)	J02012; SEQ ID NO:10 (proviral oncogene v-*myb*)	640	275-327	Kalkbrenner *et al.*, *Oncogene* (1990), 5(5):657-61.
c-*myc* (human)	X00364; SEQ ID NO:11 (human c-*myc* oncogene)	439	129-144	Sarid *et al.*, *Proc. Natl. Acad. Sci. USA* (1987), 84(1):170-3.
v-*ras* (Harvey Murine Sarcoma Virus)	M77193; SEQ ID NO:12 (Rat sarcoma virus v-*ras* oncogene)	189	32-44	Zhang *et al.*, *Science* (1990), 249:162-5 (1990)

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
v-*src* (Rous Sarcoma Virus)	U41728; SEQ ID NO:13 (RSV Schmidt-Ruppin A clone SRA-V; v-*src* gene)	526	430-433	Bryant *et al.*, *Mol. Cell. Bio.* (1984), 4(5):862-6.
c-*yes* (chicken)	D00333; SEQ ID NO:14 (human c-*yes*-2 gene)	541	438-441	Zheng *et al.*; *Oncogene* (1989), 4(1):99-104.

Engineering of Vectors for Host Cell Transfection

The engineering of vectors for expression of a particular CTG, preferably a dCTG, is based on standard methods of recombinant DNA technology, *i.e.* insertion of the dCTG via the polylinker of standard or commercially available expression vectors. The dCTG is operably linked to a strong promoter. Generally speaking, a "strong" promoter is a promoter which achieves constitutively high expression of the dCTG in the transfected cells. Each promoter should include all of the signals necessary for initiating transcription of the relevant downstream sequence. These conditions are fulfilled, for example, by the pBK-CMV expression vector available from Stratagene Cloning Systems, La Jolla, CA (catalog no. 212209). The pBK-

CMV vector contains the cytomegalovirus (CMV) immediate early promoter. dCTGs xenogenic with respect to a particular target proto-oncogene may be isolated by conventional nucleic acid probing techniques, given the availability of a highly homologous probe represented by the cognate retroviral oncogene and/or the human proto-oncogene itself.

Collection of Host Cells for Transfection

The host cells which may be transfected to derive the cellular immunogens of the present invention must express class I MHC and be susceptible to isolation and culture. Fibroblasts express class I MHC and may be cultured. Accordingly, punch biopsies of host human skin are performed to harvest fibroblasts. Punch biopsies can be performed by a competent physician as a standard clinical procedure. Each biopsy yields a starting population of 1-2 X 10^7 cells that would proliferate in culture. Methods for the preparation of tissue cultures of human fibroblasts are well developed and widely used. *See*, Cristofalo and Carpenter, *J. Tissue Culture Methods* (1980), 6:117-121, the entire disclosure of which is incorporated herein by reference. Essentially, skin obtained by punch biopsy is washed using an appropriate wash medium, finely minced and cultured in a suitable culture medium, such as Dulbecco's Modified Eagle Medium (DMEM), under CO_2 at 37°C. The cells are trypsinized with a trypsin solution and transferred to a larger vessel and incubated at 37°C in culture fluid.

Host Cell Transfection

The expression vector carrying the dCTG is used to transfect biopsied host cells according to conventional transfection methods. One method of transfection involves the addition of DEAE-dextran to increase the uptake of the naked DNA molecules by a recipient cell. *See* McCutchin and Pagano, *J. Natl. Cancer Inst.* (1968) 41:351-7. Another method of transfection is the calcium phosphate precipitation technique which depends upon the addition of Ca^{++} to a phosphate-containing DNA solution. The resulting precipitate

apparently includes DNA in association with calcium phosphate crystals. These crystals settle onto a cell monolayer; the resulting apposition of crystals and cell surface appears to lead to uptake of the DNA. A small proportion of the DNA taken up becomes expressed in a transfectant, as well as in its clonal descendants. *See* Graham *et al.*, *Virology* (1973), 52:456-467 and *Virology* (1974), 54:536-539.

Preferably, transfection is carried out by cationic phospholipid-mediated delivery. In particular, polycationic liposomes can be formed from N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) or related liposome-forming materials. *See* Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7417 (DNA-transfection); Malone *et al.*, *Proc. Natl. Acad. Sci. USA* (1989), 86:6077-6081) (RNA-transfection). One preferred technique utilizes the LipofectAMINE™ Reagent (Cat. No. 18324-012, Life Technologies, Inc., Gaithersburg, MD) which is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) (Chemical Abstracts Registry name: N-[2-({2,5-bis[(3-aminopropyl)amino]-1-oxypentyl}amino)ethyl]-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-1-propanaminium trifluoroacetate), and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. Transfection utilizing the LipofectAMINE™ Reagent is carried out according to the manufacturer's published protocol. The protocol (for Cat. No. 18324-012) provides for either transient or stable transfection, as desired.

The advantage of transient expression is its rapidity, *i.e.* there is no requirement for cellular proliferation to select for stable integration events. This rapidity could conceivably be of major clinical importance, in cases of an already metastatic tumor burden, wherein the weeks required for selection of stable transfectants may simply not be available to the clinician.

There are, nonetheless, two general disadvantages to the use of transient transfection. The first is that expression usually peters out after a few days, in contrast to the continual expression in the case of stable transfection.

This is not particularly crippling in terms of our immunization protocol. The inoculated, irradiated cells used for immunization would likely not survive *in vivo* for more than 4 or 5 days, in any case. Thus the nominal advantage accruing to stable transfection, that of a long-duration expression by the progeny of the parental inoculated cell, is not of particular relevance in the case of the immunizing regime described herein, which is based on the use of non-dividing, probably short-lived cells.

A second disadvantage of transient transfection resides in the fact that it yields a cell population, only a subset of which has actually been transfected and thus expresses the protein encoded by the transgene. This problem is obviated in the case of stable transfection, wherein over time one can develop a pure population of transfectants via selection for a resistance marker, such as *neo*, under conditions of clonal proliferation of the initial stable transfectants, *i.e.* daughter cells of transiently transfected cells lack the transgene, in contrast to the case with stable transfectants. In the situation where there is sufficient time to effect immunization based on stably transfected cells, the progeny of all transfected clones would be utilized, not just the progeny of a single clone, as is sometimes done for detailed biochemical and molecular analyses of gene expression. Clearly the more clones utilized, the more quickly one can arrive at the requisite number of cells to be used for immunization.

Percentage of Cells Exhibiting dCTG Expression

The percentage of cells exhibiting dCTG expression may be determined by an immunohistology assay. In this procedure, a small number of cells (~ 500) from the harvested pellet following centrifugation of transfected cells are deposited on a cover slip and fixed with cold acetone. At this point, a standard immunohistological assay is carried out with the cells on the cover slip, *i.e.* addition of a primary monoclonal antibody reactive to the dCTG-encoded protein, followed by the addition of a developing antibody, *e.g.* a fluorescent tagged antibody reactive to the primary monoclonal antibody.

Measurement of the percentage of cells scoring as dCTG-positive in the fluorescent assay allows a determination of the number of positive transfectants in the starting culture, and thus the number of total cells to be used for immunization to arrive at the desired number of dCTG-positive cells to be inoculated in the patient.

If, as would be almost certain, the percentage of cells scoring as dCTG-positive is less than one hundred percent, one can simply increase the number of cells to be used for immunization, so as to include the desired number of transfectants. The non-transfected cells in the immunizing population would simply represent x-irradiated, autologous fibroblasts that would constitute no danger to the patient.

Transfectant Irradiation

Prior to return to the host, the transfected cells are preferably irradiated. The transfectants are irradiated with a radiation dose sufficient to render them non-dividing, such as a dose of 25 By or 2500R. The cells are then counted by trypan blue exclusion, and about 2 X 10^7 irradiated transfectants are resuspended in a volume of 0.2-0.4 ml of Hanks Balanced Salt Solution.

Vaccination Procedure

The transfected cells are returned to the host to achieve vaccination. The cells may be reimplanted at the same body site from which they were originally harvested, or may be restored to a different site.

It is the object of the present invention to generate a systemic tumor immune response, so as to fight metastasis formation wherever any metastases are found. Accordingly, there is no reason to inject the transfected cells at the same body site from which they were taken. Intramuscular or subcutaneous inoculation at a distal site would suffice to yield a systemic response. Thus, patients are preferably vaccinated by subcutaneous inoculation of the transfected cells.

For s-*crc* overexpression associated with colon carcinoma, partial venous inoculation is preferred, as the liver is a frequent site of metastases. For vaccinating against breast cancers and lymphomas, systemic immunization is preferred.

As a general rule, it is desirable to generate the strongest immune response consistent with clinical monitoring of no adverse side effects, *i.e.* multiple rounds of inoculation with, for example 10^7 cells, at each round. The number of rounds of inoculation is selected accordingly. The efficacy of the inoculation schedule may be monitored by a delayed hypersensitivity reaction administered to the patient. A course of about up to 10 inoculations, at 2-3 week intervals, may be utilized. It may be appreciated that the inoculation schedule may be modified in view of the immunologic response of the individual patient, as determined with resort to the delayed-type hypersensitivity (DTH) reaction.

Patient Response Monitoring by Delayed-type Hypersensitivity Reaction

Patients are assessed for reactivity to the irradiated transfectants by a test of skin reactivity in a DTH reaction. DTH has been used clinically (Chang *et al.* (1993), *Cancer Research* 53:1043-1050). To measure reactivity to the autologous irradiated transfectants, 10^4 - 10^6 cells in a volume of 0.1 ml Hanks buffered saline solution (HBSS) are inoculated intradermally into the host. Induration is measured 48 hours later, as an average of two perpendicular diameters (responses of greater than ≥ 2 mm is considered positive).

One advantage to the DTH assay is that it can independently assess the induction of T cell reactivity to (i) the transfectants used for immunization (*i.e.* the set of 5 or more dCTGs chosen for immunization purposes, each containing non-self determinants) and (ii) transfectants, as transfected with the human dCTG itself containing only self determinants. Thus, the induction of reactivity to the transfectants used for immunization establishes that the immunizing transfectants are in fact immunogenic, that is, the patient has not exhibiting a much weakened capacity for immune response.

If the patient is demonstrably capable of response to the immunizing transfectants, then skin testing with the dCTG (human) transfectants would establish whether or not reactivity to the human proto-oncogene encoded product had been induced. According to the practice of the invention, inoculation of the immunizing transfectants would continue for at least as long as the induction of reactivity to the human proto-oncogene-encoded protein occurs.

The practice of the invention is illustrated by the following nonlimiting examples.

Example 1

Immunization of Chickens Against c-*src*(527)-Induced Tumors By Vaccination with v-*src* DNA

A. Genes

The oncogene c-*src*(527) is an activated form of chicken c-*src*. Its protein product $pp60^{c\text{-}src(527)}$ differs from the protein product of c-*src*, $pp60^{c\text{-}src}$, by only a single amino acid substitution, phenylalanine for tyrosine at residue 527 (Kmiecik and Shalloway, (1987) *Cell* 49, 65-73). This substitution eliminates the negative regulatory influence exerted on $pp60^{c\text{-}src}$ phosphokinase activity by the enzymatic phosphorylation of the position 527 tyrosine. The protein product of v-*src*, $pp60^{v\text{-}src}$, shows a number of sequence differences with $pp60^{c\text{-}src}$ (Takeya and Hanafusa, (1983) *Cell* 32, 881-890), including scattered single amino acid substitutions within the first 514 residues and a novel C terminus of 12 amino acids (residues 515-526), in place of the nineteen C terminal amino acids of $pp60^{c\text{-}src}$ (residues 515-533). Both the v-*src*-positive plasmid, pMv*src*, and the c-*src*(527)-positive plasmid, pc*src*527, were originally shown (Kmiecik and Shalloway, (1987) *Cell* 49, 65-73) to transform murine NIH 3T3 cells in culture. However, the v-*src*-induced transformants exhibited a more rapid or more extensive colony growth in soft agarose than the c-*src*(527)-induced transformants, as well as a usually shorter latency of tumor formation in nude mice (*id.*).

B. Plasmids

1. pvSRC-C1

The pVSRC-C1 plasmid was prepared as described by Halpern *et al.*, (1991) *Virology* 180, 857-86. Essentially, the plasmid was derived from the pRL^{v}-*src* plasmid (Halpern *et al.*, (1990) *Virology* 175, 328-331) by subcloning the v-*src*(+) *Xho*I-*Eco*RI fragment of the latter into the multiple cloning sequence of pSP65 (Melton *et al.*, (1984) *Nucleic Acids Res.* 12, 7035-7056) which had been cleaved with *Sal*I and *Eco*RI; since ligation of the *Xho*I overhang at the *Sal*I site destroys both recognition sequences, subsequent removal of the v-*src*(+) insert from the vector was achieved by digestion with *Eco*RI and with *Hind*III, which cleaves at a position in the multiple cloning sequence adjacent to the *Sal*I site. The pVSRC-C1 plasmid was restricted with *Eco*RI and *Hind*III, so as to liberate the tumorigenic insert. This insert included the v-*src* oncogene of the subgroup A strain of Prague RSV, as flanked downstream by a portion of the long terminal repeat (LTR) of RSV (from the 5' start of the LTR, to the single *Eco*RI site).

2. pMv*src*

The pMv*src* plasmid was generously provided by Dr. David Shalloway, Cornell University, Ithaca, NY. The plasmid is prepared according to Johnson *et al.*, (1985) *Mol. Cell. Biol.* 5, 1073-1083. Briefly, the 3.1-kb *Bam*HI-*Bg*/II Schmidt Ruppin A v-*src* fragment from plasmid pN4 (Iba *et al.*, (1984) *Proc. Nat. Acad. Sci. USA* 81, 4424-4428) is inserted into the pEVX plasmid (Kriegler *et al.*, (1984) *Cell* 38,483-491) at a *Bg*/II site lying between two Moloney murine leukemia virus (MoMLV) long terminal repeats (LTRs). This fragment contains 276 bp of pBR322 DNA from the pBR322 *Bam*HI to *Sal*I sites followed by 2.8 kb of Rous sarcoma virus (RSV) DNA from the *Sal*I site that is about 750 bp upstream of the *env* termination codon down to the *Nru*I site that is about 90 bp downstream of the v-*src* termination codon. (The

*Nru*I site is converted to a *Bg*/II site in the construction of pN4.) Ligation is performed by using a 10:1 insert-vector DNA fragment molar ratio.

The pMv*src* plasmid was restricted with NheI, so as to liberate a tumorigenic fragment. The fragment included the v-*src* oncogene of the subgroup A strain of Schmidt-Ruppin RSV, as flanked upstream by most of the Moloney murine leukemia virus (MoMLV) LTR (from the *Nhe*I site near the 5′ start of the LTR, to the 3′ end of this LTR) and downstream by a small portion of the MoMLV LTR (from the 5′ start to the *NheI* site).

3. pc*src*527

The pc*src*527 plasmid is prepared according to Kmiecik and Shalloway, (1987) *Cell* 49, 65-73. Briefly, a plasmid is constructed by cleaving expression vector pEVX (Kriegler *et al.*, (1984) *Cell* 38,483-491 at its unique *Bg*III site lying between two MoMLV LTRs and inserting the 3.2 kilobase (kb) pair *Bam*HI-*Bg*III hybrid *src* fragment from plasmid pHB5 in the proper orientation. This fragment contains sequences from pBR322, the SRA *env* 3′ region, SRA v-*src*, *src* from recovered ASV, and chicken c-*src*. The *Bg*III site is generated by insertion of a linker at the *Sac*I site about 20 bp downstream from the c-*src* termination codon. The restriction map of pMHB5 contains the MoMLV splice donor about 60 bp downstream from the 3′end of the upstream LTR and the v-*src* splice acceptor about 75 bp upstream from the *src* ATG.

Plasmid pMHB5527 is constructed by inserting the synthetic double-stranded DNA oligomer

```
5'        CCAGTTCCAGCCTGGAGAGAACCTATA (SEQ ID NO:1)          3'
3'  TCGGGGTCAAGGTCGGACCTCTCTTGGATATCTAG (SEQ ID NO:2)       5'
```

into pMHB5 between the *Ban*II site at c-*src* codon 524 and the downstream unique *Bg*III site. This alters the TAC Tyr 527 codon to a TTC Phe codon while preserving the remaining c-*src* coding region. Equimolar amounts of the double-stranded oligomer and three gel-purified tandem restriction fragments from pMHB5 are ligated in one reaction, which contains the following: the

oligomer with *Ban*II and *Bg*III complementary ends, the 3 kb *Bg*III-*Bg*II (*Bg*II in the pEVX ampicillin resistance gene) partial digest fragment, the adjacent 6.1 kb *Bg*II-*Bg*II (downstream Bg*II* in c-*src*) fragment, and the 0.38 kb *Bg*II-*Ban*II (*Ban*II at c-*src* codon 524) fragment.

Plasmid pc*src*527 is constructed by replacing the 2 kb *Sal*I (in *env*)-*Mlu*I (in c-*src*) fragment in plasmid pMHB5527, with the homologous fragment from plasmid p5H. This fragment contains the coding sequence for the c-*src* amino region (codons 1 to 257) that have been isolated by molecular cloning of a c-*src* provirus and previously shown by sequencing to contain authentic c-*src* sequence without the mutation at codon 63 (Levy *et al.*, (1986) *Proc. Natl. Acad. Sci. USA* 83, 4228-4232). Equimolar amounts of complementary gel-purified *Sal*I-*Mlu*I fragments from p5H and the other plasmids are ligated.

The pc*src*527 plasmid was restricted with *Nhe*I, so as to liberate a tumorigenic fragment. The tumorigenic fragment included the c-*src*(527) oncogene, as flanked by the same LTR complement as in pMv*src*.

C. Animals

Chickens of two closed lines, SC and TK, were utilized. These lines differ at the major histocompatibility (*B*) complex (B^2/B^2 for the SC line, B^{15}/B^{21} for the TK line). Embryonated eggs were obtained from Hyline International (Dallas Center, IA). All chickens were hatched at the University of New Hampshire Poultry Research Farm and housed in isolation.

D. Tumor Induction by Plasmid DNA

Tumors were induced by subcutaneous inoculation in the wing web of a *src*-positive plasmid according to the technique described by Fung *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80, 353-357 and Halpern *et al.*, (1990) *Virology* 175, 328-331. Of the three tumorigenic plasmids utilized here, all were adjusted, prior to inoculation, to a concentration of 100 μg of enzyme--restricted DNA per 100 μl of phosphate-buffered saline. The conditions of

inoculation used for particular experiments (age of chicken at time of inoculation, amount of plasmid, etc.) are indicated below.

E. Growth of Primary (wing web) Tumors in TK or SC Chickens Inoculated with pVSRC-C1, pMv*src* or pc*src*527

Individual 1-day-old chickens of line TK or of line SC were inoculated with 100 μg of either pVSRC-C1, pMv*src* or pc*src*527. The mean tumor diameter (mm) at a particular time point and for any one group of TK or SC line chickens inoculated with an individual *src*-positive construct was computed as the sum of the diameters of the primary tumors divided by the number of chickens surviving to that point. The results are shown in Fig. 1A (line TK) and Fig. 1B (line SC). The ratios at each time point show, for a particular group, the number of chickens bearing palpable tumors to the total number of survivors to that point (standard typeface for pc*src*527, italics for pVSRC-C1, bold typeface for pMV*src*). Error bars (unless obscured by the symbol) indicate standard error.

F. Growth of Challenge (wing web) Tumors in Test and Control Line TK Chickens Under Conditions of Priming and Homologous Challenge with pc*src*527, or Priming and Homologous Challenge with pVSRC-Cl

Growth of challenge (wing web) tumors in test and control line TK chickens was determined under conditions of (i) priming and homologous challenge with pc*src*527, or (ii) priming and homologous challenge with pVSRC-Cl. Test chickens were primed at 1 day posthatch with 100 μg of construct; test and control chickens were challenged at five weeks posthatch with 200 μg of construct. The mean challenge tumor diameter was computed as described in the preceding section. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated for priming and homologous challenge with pc*src*527 (Fig. 2, panel A) and priming and homologous challenge with pVSRC-Cl (Fig. 2, panel B)

(standard typeface for control group, bold typeface for test group). The statistical comparison between the mean challenge tumor diameters of the test versus the control group at a particular time point was made using a two-tailed student's t test, *($p<0.05$), **($p<0.01$), ***($p<0.001$). The statistical comparison between the ratios of chickens bearing palpable challenge tumors to total number of survivors of the test versus the control group at a particular time point was made using a chi-squared test; the paired ratios are underlined for only those time points where $p<0.05$. Error bars indicate standard error.

G. Growth of Challenge (wing web) Tumors in Test and Control line TK chickens under Conditions of Priming with pVSRC-C1 and Heterologous Challenge with pc*src*527, or Priming with pc*src*527 and Heterologous Challenge with pVSRC-Cl

Growth of challenge (wing web) tumors in test and control line TK chickens, was determined under conditions of (i) priming with pVSRC-C1 and heterologous challenge with pc*src*527, or (ii) priming with pc*src*527 and heterologous challenge with pVSRC-Cl. Test chickens were primed at 1 day posthatch with 100 μg of construct; test and control chickens were challenged at five weeks posthatch with 200 μg of construct. The mean challenge tumor diameter was computed as described in Section E. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated for priming with pVSRC-C1 and heterologous challenge with pc*src*527 (Fig. 3, panel A) and priming with pc*src*527 and heterologous challenge with pVSRC-Cl (Fig. 3, panel B) (standard typeface for control group, bold typeface for test group). Statistical comparisons were made between test and control groups at a particular time point as described in the preceding section [*($p<0.05$), **($p<0.01$), ***($p<0.001$), for the student's t test], and the paired ratios are underlined for only those time points where, in the chi-squared test, $p<0.05$. Error bars indicate standard error.

H. Discussion

In a direct comparison of the growth of tumors induced in line TK by either pMv*src* or pVSRC-C1, a similar pattern of relatively rapid regression was observed. This result established that the difference in LTR complement between these two v-*src* positive constructs did not exert a major influence on the tumor growth pattern in the TK line (Fig. 1, panel A). By contrast, much more extensive and persistent tumor growth resulted from inoculation of TK chickens with the pc*src*527 construct (Fig. 1, panel A). The relatively greater growth capacity of tumors induced by this construct indicated that in the TK line, the c-*src*(527) oncogene is much more highly tumorigenic than the v-*src* oncogene. This difference did not, however, generalize to the SC line (Fig. 1, panel B). The SC line was chosen for comparison with the TK line on the basis of earlier observations (Halpern *et al.*, (1993) *Virology* 197, 480-484) that v-*src* DNA-induced tumors engender a much weaker tumor immune response in line SC than in line TK. Whereas the growth of pc*src*527-induced primary tumors was virtually indistinguishable in the two lines, the growth of the v-*src*-induced tumors was considerably greater in the SC than in the TK line (Fig. 1). Thus v-*src*, but not c-*src*(527), gives rise to primary tumors whose growth patterns differ in the two lines analyzed here.

Only minimal protection against homologous challenge was observed under conditions of priming to c-*src*(527) DNA, indicative of the induction of a relatively weak tumor immune response (Fig. 2, panel A; a statistically significant lowering of challenge tumor growth in the test versus the control chickens was observed at only one time point). By contrast, the v-*src* DNA-primed chickens showed excellent protection against the homologous tumor challenge (Fig. 2, panel B).

Priming with v-*src* DNA engenders a relatively greater degree of protection against challenge with c-*src*(527) DNA, than that afforded by priming with c-*src*(527) DNA itself (Fig. 3, panel A). The degree of protection was weaker than that determined (Fig. 2, panel B) for the case of priming and homologous challenge with v-*src* DNA. Only marginal protection was

observed, however, when the heterologous challenge protocol was carried out in the reverse order (Fig. 3, panel B). These results demonstrate that induction of reactivity to an antigenicity specified in tumor cells by an overexpressed proto-oncogene can confers tumor immunity.

Example 2

Vaccination Protocol

The following is a representative vaccination protocol according to the present invention.

A. Skin Punch Biopsy

A punch biopsy of skin is obtained by a trained physician following standard medical practice.

B. Preparation of Primary Fibroblast Culture

Under sterile conditions, the skin obtained by punch biopsy is put in a tube with 10 ml of the following wash medium: Dulbecco's Modified Eagle Medium (DMEM), containing sodium bicarbonate (30 ml/liter of a 5.6% solution) and penicillin/streptomycin (2 ml/liter of a pen-strep stock solution containing 5000 units penicillin and 5000 μg of streptomycin/ml, pH 7.2-7.4.). In a sterile hood, the skin biopsy is added to a Petri dish, and then transferred several times to new Petri dishes containing the same wash medium. The biopsy is then finely minced with two scalpels, and 2-4 pieces (<1 mm^3) of the minced biopsied are placed in the middle part of one or more T25 flasks. The flask is placed in a tissue culture incubator at 37°C for one half hour with the cap firmly closed, then opened for 10 minutes. The following culture medium is prepared: DMEM containing sodium bicarbonate; antibiotics; and 10% fetal calf serum containing 2.5 μg/ml fungizone, 40 μg/ml gentamicin, and 1% glutamine(3% W/V). Two ml of the culture medium is then added to the flask, and the flask is incubated at 37°C (5% CO_2), with the cap lightly unscrewed. The flask is left for three days without moving so as to obtain adhesion of the

separate pieces of skin to the plastic. Afterwards, the medium is changed two times per week over a 3-4 week period always adding 2-3 ml of medium. To trypsinize the skin cell culture, one needs zones of confluence. After aspirating the culture medium, 5 ml of the Puck's Saline A/EDTA solution (0.4 g EDTA to 1 liter of Puck's Solution A) is added and immediately aspirated. Then 1 ml of trypsin solution (0.05/0.02% trypsin in PBS, without Ca++ or Mg++) is added and incubated for 5 min at 37°C, at which time 2 ml of culture fluid is added to stop the action of the trypsin. The cells are then transferred to a larger flask (T75) and incubated at 37°C in 15 ml of culture fluid, which is changed every 2 days.

C. Fibroblast Transfection

The fibroblasts (2 X 10^5 cells) are washed twice in DMEM without serum or antibiotics. A LipofectAMINE™-DNA solution is prepared by mixing in tube #1 mix 400μl DMEM and 10μl of dCTG vector DNA (1μg/ul). In tube #2, 400 μl DMEM and 25 Ml of LipofectAMINE Reagent (Life Technologies, cat. no. 18324-012) are mixed. The contents of tube #1 and #2 are mixed together and are then left sitting at room temperature for 30 hours. Then, 3.2 ml of the LipofectAMINE™-DNA solution is added to the cells. The cells are incubated for six hours at 37°C, washed once with Hank's Balanced Salt Solution, and then refed with growth medium and incubated for an additional 24 hours at 37°C

D. Transfectant Irradiation

Transfectants are irradiated to a dose of 25 By or 2500R. the cells are then counted by trypan blue exclusion. 2 X 10^7 irradiated transfectants are resuspended in a volume of 0.2-0.4 ml of Hanks Balanced Salt Solution.

E. Vaccination

Patients are vaccinated by subcutaneous inoculation of 2 X 10^7 irradiated cells at 2-3 week intervals. A shorter or longer regimen is used,

depending upon the results of delayed type hypersensitivity (DTH) reaction monitoring (described below).

F. Patient Assessment by DTH Monitoring

Patients are assessed for reactivity to the irradiated transfectants by a test of skin reactivity in a DTH reaction, as described by Chang *et al.* (1993), *Cancer Research* 53:1043-1050. To measure reactivity to the autologous irradiated transfectants, 10^4 - 10^6 transfected irradiated cells in a volume of 0.1 ml HBSS are inoculated intradermally. Induration is measured 48 hours later, as an average of two perpendicular diameters. Responses of greater than 2 mm are considered positive.

Example 3

v-*myc* Transfection of Murine Fibroblasts

A. Vector Preparation

The v-*myc* retroviral oncogene of avian myelocytomatosis virus MC29 (Land *et al.* (1983), *Nature* 304:596-602) was obtained from the American Type Culture Collection, Rockville, MD, 20852, as the pSVv-*myc* vector (ATCC No. 45014). The v-*myc*-positive *Eco*RI-*Kpn*I fragment of pSV*v-myc* was ligated into the polylinker sites of the pBK-CMV plasmid (Stratagene Cloning Systems, La Jolla, CA).

B. Cell Transfection

Stable transfection using the pBK-CMV-v-*myc* vector was carried out on a line of A31 fibroblasts (Balb/c origin), obtained from the ATCC. 2 X 10^5 cells were seeded in a 100 mm/dish and allowed to grow for 18-20 h (RPMI 1640 medium and 10% fetal bovine serum), at which time the cells reached 50-70% confluence. The cells were then washed twice in Dulbecco's Modified Eagles Medium (without serum or antibiotics). A LipofectAMINE™-DNA solution was prepared according to Example 2.C., with the pBK-CMV-v-

myc vector DNA, and 3.2 ml of the LipofectAMINE™-DNA solution added to the cells. The cells were then incubated for 6 hours at 37°C, washed once with Hank's Balanced Salt Solution, and then refed with the growth medium and incubated for an additional 24 hour at 37°C. Thereafter, the cells were fed once every two days with growth medium containing 250 μg/ml geneticin (G418; Gibco BRL cat. no. 11811) as the selective marker. Within two weeks, colonies were picked and expanded into permanent cell lines. The cells were then washed and collected by centrifugation.

It should be noted that the procedure for transient transfection is the same, through the point of incubation with the Lipofectamine™-DNA solution. Thereafter, the cells are washed and incubated for 72 hours in growth medium.

All references cited with respect to synthetic, preparative and analytical procedures are incorporated herein by reference.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indication the scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Allegheny University of the Health Sciences
Halpern, Michael S.
England, James M.

(ii) TITLE OF INVENTION: CANCER VACCINE

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Seidel, Gonda, Lavorgna & Monaco, P.C.
(B) STREET: Suite 1800, Two Penn Center Plaza
(C) CITY: Philadelphia
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19102

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/010,262
(B) FILING DATE: 19-JAN-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Monaco, Daniel A.
(B) REGISTRATION NUMBER: 30,480
(C) REFERENCE/DOCKET NUMBER: 7933-33 PC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (215) 568-8383
(B) TELEFAX: (215) 568-5549

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCAGTTCCAG CCTGGAGAGA ACCTATA 27

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATCTATAGG TTCTCTCCAG GCTGGAACTG GGGCT 35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1599 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAGACTGTGC CCTGTCCACG GTGCCTCCTG CATGTCCTGC TGCCCTGAGC TGTCCCGAGC 60

TAGGTGACAG CGTACCACGC TGCCACCATG AATGAGGTGT CTGTCATCAA AGAAGGCTGG 120

CTCCACAAGC GTGGTGAATA CATCAAGACC TGGAGGCCAC GGTACTTCCT GCTGAAGAGC 180

GACGGCTCCT TCATTGGGTA CAAGGAGAGG CCCGAGGCCC CTGATCAGAC TCTACCCCCC 240

TTAAACAACT TCTCCGTAGC AGAATGCCAG CTGATGAAGA CCGAGAGGCC GCGACCCAAC 300

ACCTTTGTCA TACGCTGCCT GCAGTGGACC ACAGTCATCG AGAGGACCTT CCACGTGGAT 360

TCTCCAGACG AGAGGGAGGA GTGGATGCGG GCCATCCAGA TGGTCGCCAA CAGCCTCAAG 420

CAGCGGGCCC CAGGCGAGGA CCCCATGGAC TACAAGTGTG GCTCCCCCAG TGACTCCTCC 480

ACGACTGAGG AGATGGAAGT GGCGGTCAGC AAGGCACGGG CTAAAGTGAC CATGAATGAC 540

TTCGACTATC TCAAACTCCT TGGCAAGGGA ACCTTTGGCA AAGTCATCCT GGTGCGGGAG 600

AAGGCCACTG GCCGCTACTA CGCCATGAAG ATCCTGCGAA AGGAAGTCAT CATTGCCAAG 660

GATGAAGTCG CTCACACAGT CACCGAGAGC CGGGTCCTCC AGAACACCAG GCACCCGTTC 720

CTCACTGCGC TGAAGTATGC CTTCCAGACC CACGACCGCC TGTGCTTTGT GATGGAGTAT 780

GCCAACGGGG GTGAGCTGTT CTTCCACCTG TCCCGGGAGC GTGTCTTCAC AGAGGAGCGG 840

GCCCGGTTTT ATGGTGCAGA GATTGTCTCG GCTCTTGAGT ACTTGCACTC GCGGGACGTG 900

GTATACCGCG ACATCAAGCT GGAAAACCTC ATGCTGGACA AAGATGGCCA CATCAAGATC 960

ACTGACTTTG GCCTCTGCAA AGAGGGCATC AGTGACGGGG CCACCATGAA AACCTTCTGT 1020

GGGACCCCGG AGTACCTGGC GCCTGAGGTG CTGGAGGACA ATGACTATGG CCGGGCCGTG 1080

GACTGGTGGG GGCTGGGTGT GGTCATGTAC GAGATGATGT GCGGCCGCCT GCCCTTCTAC 1140

AACCAGGACC ACGAGCGCCT CTTCGAGCTC ATCCTCATGG AAGAGATCCG CTTCCCGCGC 1200

ACGCTCAGCC CCGAGGCCAA GTCCCTGCTT GCTGGGCTGC TTAAGAAGGA CCCCAAGCAG 1260

AGGCTTGGTG GGGGGCCCAG CGATGCCAAG GAGGTCATGG AGCACAGGTT CTTCCTCAGC 1320

ATCAACTGGC AGGACGTGGT CCAGAAGAAG CTCCTGCCAC CCTTCAAACC TCAGGTCACG 1380

TCCGAGGTCG ACACAAGGTA CTTCGATGAT GAATTTACCG CCCAGTCCAT CACAATCACA 1440

CCCCCTGACC GCTATGACAG CCTGGGCTTA CTGGAGCTGG ACCAGCGGAC CCACTTCCCC 1500

CAGTTCTCCT ACTCGGCCAG CATCCGCGAG TGAGCAGTCT GCCCACGCAG AGGACGCACG 1560

CTCGCTGCCA TCACCGCTGG GTGGTTTTTT ACCCCTGCC 1599

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4530 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATTCTCGAG CTCGTCGACC GGTCGACGAG CTCGAGGGTC GACGAGCTCG AGGGCGCGCG 60

CCCGGCCCCC ACCCCTCGCA GCACCCCGCG CCCCGCGCCC TCCCAGCCGG GTCCAGCCGG 120

AGCCATGGGG CCGGAGCCGC AGTGAGCACC ATGGAGCTGG CGGCCTTGTG CCGCTGGGGG 180

CTCCTCCTCG CCCTCTTGCC CCCCGGAGCC GCGAGCACCC AAGTGTGCAC CGGCACAGAC 240

ATGAAGCTGC GGCTCCCTGC CAGTCCCGAG ACCCACCTGG ACATGCTCCG CCACCTCTAC 300

CAGGGCTGCC AGGTGGTGCA GGGAAACCTG GAACTCACCT ACCTGCCCAC CAATGCCAGC 360

CTGTCCTTCC TGCAGGATAT CCAGGAGGTG CAGGGCTACG TGCTCATCGC TCACAACCAA 420

GTGAGGCAGG TCCCACTGCA GAGGCTGCGG ATTGTGCGAG GCACCCAGCT CTTTGAGGAC 480

AACTATGCCC TGGCCGTGCT AGACAATGGA GACCCGCTGA ACAATACCAC CCCTGTCACA 540

GGGGCCTCCC CAGGAGGCCT GCGGGAGCTG CAGCTTCGAA GCCTCACAGA GATCTTGAAA 600

GGAGGGGTCT TGATCCAGCG GAACCCCCAG CTCTGCTACC AGGACACGAT TTTGTGGAAG 660

GACATCTTCC ACAAGAACAA CCAGCTGGCT CTCACACTGA TAGACACCAA CCGCTCTCGG 720

GCCTGCCACC CCTGTTCTCC GATGTGTAAG GGCTCCCGCT GCTGGGGAGA GAGTTCTGAG 780

GATTGTCAGA GCCTGACGCG CACTGTCTGT GCCGGTGGCT GTGCCCGCTG CAAGGGGCCA 840

CTGCCCACTG ACTGCTGCCA TGAGCAGTGT GCTGCCGGCT GCACGGGCCC CAAGCACTCT 900

GACTGCCTGG CCTGCCTCCA CTTCAACCAC AGTGGCATCT GTGAGCTGCA CTGCCCAGCC 960

CTGGTCACCT ACAACACAGA CACGTTTGAG TCCATGCCCA ATCCCGAGGG CCGGTATACA 1020

TTCGGCGCCA GCTGTGTGAC TGCCTGTCCC TACAACTACC TTTCTACGGA CGTGGGATCC 1080

TGCACCCTCG TCTGCCCCCT GCACAACCAA GAGGTGACAG CAGAGGATGG AACACAGCGG 1140

TGTGAGAAGT GCAGCAAGCC CTGTGCCCGA GTGTGCTATG GTCTGGGCAT GGAGCACTTG 1200

CGAGAGGTGA GGGCAGTTAC CAGTGCCAAT ATCCAGGAGT TTGCTGGCTG CAAGAAGATC 1260

TTTGGGAGCC TGGCATTTCT GCCGGAGAGC TTTGATGGGG ACCCAGCCTC CAACACTGCC 1320

CCGCTCCAGC CAGAGCAGCT CCAAGTGTTT GAGACTCTGG AAGAGATCAC AGGTTACCTA 1380

TACATCTCAG CATGGCCGGA CAGCCTGCCT GACCTCAGCG TCTTCCAGAA CCTGCAAGTA 1440

ATCCGGGGAC GAATTCTGCA CAATGGCGCC TACTCGCTGA CCCTGCAAGG GCTGGGCATC 1500

AGCTGGCTGG GGCTGCGCTC ACTGAGGGAA CTGGGCAGTG GACTGGCCCT CATCCACCAT 1560

AACACCCACC TCTGCTTCGT GCACACGGTG CCCTGGGACC AGCTCTTTCG GAACCCGCAC 1620

CAAGCTCTGC TCCACACTGC CAACCGGCCA GAGGACGAGT GTGTGGGCGA GGGCCTGGCC 1680

TGCCACCAGC TGTGCGCCCG AGGGCACTGC TGGGGTCCAG GGCCCACCCA GTGTGTCAAC 1740

TGCAGCCAGT TCCTTCGGGG CCAGGAGTGC GTGGAGGAAT GCCGAGTACT GCAGGGGCTC 1800

CCCAGGGAGT ATGTGAATGC CAGGCACTGT TTGCCGTGCC ACCCTGAGTG TCAGCCCCAG 1860

AATGGCTCAG TGACCTGTTT TGGACCGGAG GCTGACCAGT GTGTGGCCTG TGCCCACTAT 1920

AAGGACCCTC CCTTCTGCGT GGCCCGCTGC CCCAGCGGTG TGAAACCTGA CCTCTCCTAC 1980

ATGCCCATCT GGAAGTTTCC AGATGAGGAG GGCGCATGCC AGCCTTGCCC CATCAACTGC 2040

ACCCACTCCT GTGTGGACCT GGATGACAAG GGCTGCCCCG CCGAGCAGAG AGCCAGCCCT 2100

CTGACGTCCA TCGTCTCTGC GGTGGTTGGC ATTCTGCTGG TCGTGGTCTT GGGGGTGGTC 2160

TTTGGGATCC TCATCAAGCG ACGGCAGCAG AAGATCCGGA AGTACACGAT GCGGAGACTG 2220

CTGCAGGAAA CGGAGCTGGT GGAGCCGCTG ACACCTAGCG GAGCGATGCC CAACCAGGCG 2280

CAGATGCGGA TCCTGAAAGA GACGGAGCTG AGGAAGGTGA AGGTGCTTGG ATCTGGCGCT 2340

TTTGGCACAG TCTACAAGGG CATCTGGATC CCTGATGGGG AGAATGTGAA AATTCCAGTG 2400

GCCATCAAAG TGTTGAGGGA AAACACATCC CCCAAAGCCA ACAAAGAAAT CTTAGACGAA 2460

GCATACGTGA TGGCTGGTGT GGGCTCCCCA TATGTCTCCC GCCTTCTGGG CATCTGCCTG 2520

ACATCCACGG TGCAGCTGGT GACACAGCTT ATGCCCTATG GCTGCCTCTT AGACCATGTC 2580

CGGGAAAACC GCGGACGCCT GGGCTCCCAG GACCTGCTGA ACTGGTGTAT GCAGATTGCC 2640

AAGGGGATGA GCTACCTGGA GGATGTGCGG CTCGTACACA GGGACTTGGC CGCTCGGAAC 2700

GTGCTGGTCA AGAGTCCCAA CCATGTCAAA ATTACAGACT TCGGGCTGGC TCGGCTGCTG 2760

GACATTGACG AGACAGAGTA CCATGCAGAT GGGGGCAAGG TGCCCATCAA GTGGATGGCG 2820

CTGGAGTCCA TTCTCCGCCG GCGGTTCACC CACCAGAGTG ATGTGTGGAG TTATGGTGTG 2880

ACTGTGTGGG AGCTGATGAC TTTTGGGGCC AAACCTTACG ATGGGATCCC AGCCCGGGAG 2940

ATCCCTGACC TGCTGGAAAA GGGGGAGCGG CTGCCCCAGC CCCCCATCTG CACCATTGAT 3000

GTCTACATGA TCATGGTCAA ATGTTGGATG ATTGACTCTG AATGTCGGCC AAGATTCCGG 3060

GAGTTGGTGT CTGAATTCTC CCGCATGGCC AGGGACCCCC AGCGCTTTGT GGTCATCCAG 3120

AATGAGGACT TGGGCCCAGC CAGTCCCTTG GACAGCACCT TCTACCGCTC ACTGCTGGAG 3180

GACGATGACA TGGGGGACCT GGTGGATGCT GAGGAGTATC TGGTACCCCA GCAGGGCTTC 3240

TTCTGTCCAG ACCCTGCCCC GGGCGCTGGG GGCATGGTCC ACCACAGGCA CCGCAGCTCA 3300

TCTACCAGGA GTGGCGGTGG GGACCTGACA CTAGGGCTGG AGCCCTCTGA AGAGGAGGCC 3360

CCCAGGTCTC CACTGGCACC CTCCGAAGGG GCTGGCTCCG ATGTATTTGA TGGTGACCTG 3420

GGAATGGGGG CAGCCAAGGG GCTGCAAAGC CTCCCCACAC ATGACCCCAG CCCTCTACAG 3480

CGGTACAGTG AGGACCCCAC AGTACCCCTG CCCTCTGAGA CTGATGGCTA CGTTGCCCCC 3540

CTGACCTGCA GCCCCCAGCC TGAATATGTG AACCAGCCAG ATGTTCGGCC CCAGCCCCCT 3600

TCGCCCCGAG AGGGCCCTCT GCCTGCTGCC CGACCTGCTG GTGCCACTCT GGAAAGGGCC 3660

AAGACTCTCT CCCCAGGGAA GAATGGGGTC GTCAAAGACG TTTTTGCCTT TGGGGGTGCC 3720

GTGGAGAACC CCGAGTACTT GACACCCCAG GGAGGAGCTG CCCCTCAGCC CCACCCTCCT 3780

CCTGCCTTCA GCCCAGCCTT CGACAACCTC TATTACTGGG ACCAGGACCC ACCAGAGCGG 3840

GGGGCTCCAC CCAGCACCTT CAAAGGGACA CCTACGGCAG AGAACCCAGA GTACCTGGGT 3900

CTGGACGTGC CAGTGTGAAC CAGAAGGCCA AGTCCGCAGA AGCCCTGATG TGTCCTCAGG 3960

GAGCAGGGAA GGCCTGACTT CTGCTGGCAT CAAGAGGTGG GAGGGCCCTC CGACCACTTC 4020

CAGGGGAACC TGCCATGCCA GGAACCTGTC CTAAGGAACC TTCCTTCCTG CTTGAGTTCC 4080

CAGATGGCTG GAAGGGGTCC AGCCTCGTTG GAAGAGGAAC AGCACTGGGG AGTCTTTGTG 4140

GATTCTGAGG CCCTGCCCAA TGAGACTCTA GGGTCCAGTG GATGCCACAG CCCAGCTTGG 4200

CCCTTTCCTT CCAGATCCTG GGTACTGAAA GCCTTAGGGA AGCTGGCCTG AGAGGGGAAG 4260

CGGCCCTAAG GGAGTGTCTA AGAACAAAAG CGACCCATTC AGAGACTGTC CCTGAAACCT 4320

AGTACTGCCC CCCATGAGGA AGGAACAGCA ATGGTGTCAG TATCCAGGCT TTGTACAGAG 4380

TGCTTTTCTG TTTAGTTTTT ACTTTTTTTG TTTTGTTTTT TTAAAGACGA AATAAAGACC 4440

CAGGGGAGAA TGGGTGTTGT ATGGGGAGGC AAGTGTGGGG GGTCCTTCTC CACACCCACT 4500

TTGTCCATTT GCAAATATAT TTTGGAAAAC 4530

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 891 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA 60

GCTTCGGAAC AAGAGACCCT GGATCTTGAT GCTGGTGTAA GTGAACATTC AGGTGATTGG 120

TTGGATCAGG ATTCAGTTTC AGATCAGTTT AGTGTAGAAT TTGAAGTTGA ATCTCTCGAC 180

TCAGAAGATT ATAGCCTTAG TGAAGAAGGA CAAGAACTCT CAGATGAAGA TGATGAGGTA 240

TATCAAGTTA CTGTGTATCA GGCAGGGGAG AGTGATACAG ATTCATTTGA AGAAGATCCT 300

GAAATTTCCT TAGCTGACTA TTGGAAATGC ACTTCATGCA ATGAAATGAA TCCCCCCCTT 360

CCATCACATT GCAACAGATG TTGGGCCCTT CGTGAGAATT GGCTTCCTGA AGATAAAGGG 420

AAAGATAAAG GGGAAATCTC TGAGAAAGCC AAACTGGAAA ACTCAACACA AGCTGAAGAG 480

GGCTTTGATG TTCCTGATTG TAAAAAAACT ATAGTGAATG ATTCCAGAGA GTCATGTGTT 540

GAGGAAAATG ATGATAAAAT TACACAAGCT TCACAATCAC AAGAAAGTGA AGACTATTCT 600

CAGCCATCAA CTTCTAGTAG CATTATTTAT AGCAGCCAAG AAGATGTGAA AGAGTTTGAA 660

AGGGAAGAAA CCCAAGACAA AGAAGAGAGT GTGGAATCTA GTTTGCCCCT TAATGCCATT 720

GAACCTTGTG TGATTTGTCA AGGTCGACCT AAAAATGGTT GCATTGTCCA TGGCAAAACA 780

GGACATCTTA TGGCCTGCTT TACATGTGCA AAGAAGCTAA AGAAAAGGAA TAAGCCCTGC 840

CCAGTATGTA GACAACCAAT TCAAATGATT GTGCTAACTT ATTTCCCCTA G 891

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 657 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA 60

GCTTCGGAAC AAGAGACCCT GGACTATTGG AAATGCACTT CATGCAATGA AATGAATCCC 120

CCCCTTCCAT CACATTGCAA CAGATGTTGG GCCCTTCGTG AGAATTGGCT TCCTGAAGAT 180

AAAGGGAAAG ATAAAGGGGA AATCTCTGAG AAAGCCAAAC TGGAAAACTC AACACAAGCT 240

GAAGAGGGCT TTGATGTTCC TGATTGTAAA AAAACTATAG TGAATGATTC CAGAGAGTCA 300

TGTGTTGAGG AAAATGATGA TAAAATTACA CAAGCTTCAC AATCACAAGA AAGTGAAGAC 360

TATTCTCAGC CATCAACTTC TAGTAGCATT ATTTATAGCA GCCAAGAAGA TGTGAAAGAG 420

TTTGAAAGGG AAGAAACCCA AGACAAAGAA GAGAGTGTGG AATCTAGTTT GCCCCTTAAT 480

GCCATTGAAC CTTGTGTGAT TTGTCAAGGT CGACCTAAAA ATGGTTGCAT TGTCCATGGC 540

AAAACAGGAC ATCTTATGGC CTGCTTTACA TGTGCAAAGA AGCTAAAGAA AAGGAATAAG 600

CCCTGCCCAG TATGTAGACA ACCAATTCAA ATGATTGTGC TAACTTATTT CCCCTAG 657

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 966 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA 60

GCTTCGGAAC AAGAGACCCT GGTTAGACCA AAGCCATTGC TTTTGAAGTT ATTAAAGTCT 120

GTTGGTGCAC AAAAAGACAC TTATACTATG AAAGAGGATC TTGATGCTGG TGTAAGTGAA 180

CATTCAGGTG ATTGGTTGGA TCAGGATTCA GTTTCAGATC AGTTTAGTGT AGAATTTGAA 240

GTTGAATCTC TCGACTCAGA AGATTATAGC CTTAGTGAAG AAGGACAAGA ACTCTCAGAT 300

GAAGATGATG AGGTATATCA AGTTACTGTG TATCAGGCAG GGGAGAGTGA TACAGATTCA 360

TTTGAAGAAG ATCCTGAAAT TTCCTTAGCT GACTATTGGA AATGCACTTC ATGCAATGAA 420

ATGAATCCCC CCCTTCCATC ACATTGCAAC AGATGTTGGG CCCTTCGTGA GAATTGGCTT 480

CCTGAAGATA AAGGGAAAGA TAAAGGGGAA ATCTCTGAGA AAGCCAAACT GGAAAACTCA 540

ACACAAGCTG AAGAGGGCTT TGATGTTCCT GATTGTAAAA AAACTATAGT GAATGATTCC 600

AGAGAGTCAT GTGTTGAGGA AAATGATGAT AAAATTACAC AAGCTTCACA ATCACAAGAA 660

AGTGAAGACT ATTCTCAGCC ATCAACTTCT AGTAGCATTA TTTATAGCAG CCAAGAAGAT 720

GTGAAAGAGT TTGAAAGGGA AGAAACCCAA GACAAAGAAG AGAGTGTGGA ATCTAGTTTG 780

CCCCTTAATG CCATTGAACC TTGTGTGATT TGTCAAGGTC GACCTAAAAA TGGTTGCATT 840

GTCCATGGCA AAACAGGACA TCTTATGGCC TGCTTTACAT GTGCAAAGAA GCTAAAGAAA 900

AGGAATAAGC CCTGCCCAGT ATGTAGACAA CCAATTCAAA TGATTGTGCT AACTTATTTC 960

CCCTAG 966

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 399 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA 60

GCTTCGGAAC AAGAGACCCT GGTTAGACAA GAAAGTGAAG ACTATTCTCA GCCATCAACT 120

TCTAGTAGCA TTATTTATAG CAGCCAAGAA GATGTGAAAG AGTTTGAAAG GGAAGAAACC 180

CAAGACAAAG AAGAGAGTGT GGAATCTAGT TTGCCCCTTA ATGCCATTGA ACCTTGTGTG 240

ATTTGTCAAG GTCGACCTAA AAATGGTTGC ATTGTCCATG GCAAAACAGG ACATCTTATG 300

GCCTGCTTTA CATGTGCAAA GAAGCTAAAG AAAAGGAATA AGCCCTGCCC AGTATGTAGA 360

CAACCAATTC AAATGATTGT GCTAACTTAT TTCCCCTAG 399

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 309 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA 60

GCTTCGGAAC AAGAGACCCT GGTTAGACCA AAGCCATTGC TTTTGAAGTT ATTAAAGTCT 120

GTTGGTGCAC AAAAAGACAC TTATACTATG AAAGAGGTTC TTTTTTATCT TGGCCAGTAT 180

ATTATGACTA AACGATTATA TGATGAGAAG CAACAACATA TTGTAAATGA TTGTGCTAAC 240

TTATTTCCCC TAGTTGACCT GTCTATAAGA GAATTATATA TTTCTAACTA TATAACCCTA 300

GGAATTTAG 309

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1897 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CACAGATAAG GTTATTTGGG TACCCTCTCG AAAAGTTAAA CCGGACATCG CCCAAAAGGA 60

TGAGGTGACT AAGAAAGATG AGGCGAGCCC TCTTTTTGCA GGCTGGAGGC ACATAGATAA 120

GAGAATTATC ACTCTACATT CATCTTTCTC AAAGATTAAT CTACTTGTGT GTTTTATATT 180

TCATTAGAAT CGGACAGATG TTCAGTGCCA GCACCGGTGG CAGAAAGTAT TAAACCCAGA 240

ACTTAACAAA GGTCCATGGA CTAAAGAGGA GGATCAAAGG GTAATAGAAC ACGTGCAGAA 300

ATACGGTCCA AAGCGCTGGT CGGACATTGC TAAGCATTTG AAGGGAAGGA TTGGAAAACA 360

GTGCAGGGAG AGGTGGCACA ACCATCTGAA TCCAGAAGTG AAGAAAACCT CCTGGACAGA 420

AGAGGAAGAT AGAATTATTT ACCAGGCACA CAAGAGACTG GGAAACAGAT GGGCAGAAAT 480

TGCAAAGTTG CTGCCTGGAC GGACTGATAA CGCTGTCAAG AACCACTGGA ATTCCACCAT 540

GCGCCGGAAG GTCGAGCAGG AGGGTTACCC GCAGGAGTCC TCCAAAGCCG GCCCGCCCTC 600

GGCAACCACC GGCTTCCAGA AGAGCAGCCA TCTGATGGCC TTTGCCCACA ACCCACCTGC 660

AGGCCCGCTC CCGGGGGCCG GCCAGGCCCC TCTGGGCAGT GACTACCCCT ACTACCACAT 720

TGCTGAGCCA CAAAATGTCC CTGGTCAGAT CCCATATCCA GTAGCACTGC ATATAAATAT 780

TATCAATGTT CCTCAGCCAG CTGCTGCAGC TATTCAGAGA CACTATACTG ATGAAGACCC 840

TGAGAAAGAA AAACGAATAA AGGAATTAGA GTTGCTACTT ATGTCGACTG AGAATGAACT 900

GAAAGGGCAG CAGGCATTAC CAACACAGAA CCACACAGCA AACTACCCCG GCTGGCACAG 960

CACCACGGTT GCTGACAATA CCAGGACCAG TGGTGACAAT GCGCCTGTTT CCTGTTTGGG 1020

GGAACATCAC CACTGTACTC CATCTCCACC AGTGGATCAT GGTTGCTTAC CTGAGGAAAG 1080

TGCGTCCCCC GCACGGTGCA TGATTGTTCA CCAGAGCAAC ATCCTGGATA ATGTTAAGAA 1140

TCTCTTAGAA TTTGCAGAAA CACTCCAGTT AATAGACTCC TTCTTAAACA CATCGTCCAA 1200

TCACGAGAAT CTGAACCTGG ACAACCCTGC ACTAACCTCC ACGCCAGTGT GTGGCCACAA 1260

GATGTCTGTT ACCACCCCAT TCCACAAGGA CCAGACTTTC ACTGAATACA GGAAGATGCA 1320

CGGCGGAGCA GTCTAGAGCT CAATTATAAT AATCTTGCGA ATCGGGCTGT AACGGGGCAA 1380

GGCTTGACCG AGGGGACTAT AACATGTATA GGCGAAAAGC GGGGTCTCGG TTGTAACGCG 1440

CTTAGGAAGT CCCCTCGAGG TATGGCAGAT ATGCTTTTGC ATAGGGAGGG GGAAATGTAG 1500

TCTTAATCGT AGGTTAACAT GTATATTACC AAATAAGGGA ATCGCCTGAT GCACCAAATA 1560

AGGTATTATA TGATCCCATT GGTGGTGAAG GAGCGACCTG AGGGCATATG GGCGTTAACA 1620

GAACTGTCTG TCCTTGCGTC ATTCCTCATC GGATCATGTA CGCGGCAGAG TATGATTGGA 1680

TAACAGGATG GCACCATTCA TCGTGGCGCA TGCTGATTGG TGCGACTAAG GAGTTGTGTA 1740

ACCCACGAAT GTACTTAAGC TTGTAGTTGC TAACAATAAA GTGCCATTCT ACCTCTCACC 1800

ACATTGGTGT GCACCTGGGT TGATGGCCGG ACCGTCGATT CCCTGACGAC TGCGAACACC 1860

TGAATGAAGC TGAAGGCTTC AGGTACCCTT ACTTGAT 1897

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8082 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCTTGTTTG GCCGTTTTAG GGTTTGTTGG AATTTTTTTT TCGTCTATGT ACTTGTGAAT 60

TATTTCACGT TTGCCATTAC CGGTTCTCCA TAGGGTGATG TTCATTAGCA GTGGTGATAG 120

GTTAATTTTC ACCATCTCTT ATGCGGTTGA ATAGTCACCT CTGAACCACT TTTTCCTCCA 180

GTAACTCCTC TTTCTTCGGA CCTTCTGCAG CCAACCTGAA AGAATAACAA GGAGGTGGCT 240

GGAAACTTGT TTTAAGGAAC CGCCTGTCCT TCCCCCGCTG GAAACCTTGC ACCTCGGACG 300

CTCCTGCTCC TGCCCCCACC TGACCCCCGC CCTCGTTGAC ATCCAGGCGC GATGATCTCT 360

GCTGCCAGTA GAGGGCACAC TTACTTTACT TTCGCAAACC TGAACGCGGG TGCTGCCCAG 420

AGAGGGGGCG GAGGGAAAGA CGCTTTGCAG CAAAATCCAG CATAGCGATT GGTTGCTCCC 480

CGCGTTTGCG GCAAAGGCCT GGAGGCAGGA GTAATTTGCA ATCCTTAAAG CTGAATTGTG 540

CAGTGCATCG GATTTGGAAG CTACTATATT CACTTAACAC TTGAACGCTG AGCTGCAAAC 600

TCAACGGGTA ATAACCCATC TTGAACAGCG TACATGCTAT ACACACACCC CTTTCCCCCG 660

AATTGTTTTC TCTTTTGGAG GTGGTGGAGG GAGAGAAAAG TTTACTTAAA ATGCCTTTGG 720

GTGAGGGACC AAGGATGAGA AGAATGTTTT TTGTTTTTCA TGCCGTGGAA TAACACAAAA 780

TAAAAAATCC CGAGGGAATA TACATTATAT ATTAAATATA GATCATTTCA GGGAGCAAAC 840

AAATCATGTG TGGGGCTGGG CAACTAGCTG AGTCGAAGCG TAAATAAAAT GTGAATACAC 900

GTTTGCGGGT TACATACAGT GCACTTTCAC TAGTATTCAG AAAAAATTGT GAGTCAGTGA 960

ACTAGGAAAT TAATGCCTGG AAGGCAGCCA AATTTTAATT AGCTCAAGAC TCCCCCCCCC 1020

CCCCAAAAAA AGGCACGGAA GTAATACTCC TCTCCTCTTC TTTGATCAGA ATCGATGCAT 1080

TTTTTGTGCA TGACCGCATT TCCAATAATA AAAGGGGAAA GAGGACCTGG AAAGGAATTA 1140

AACGTCCGGT TTGTCCGGGG AGGAAAGAGT TAACGGTTTT TTTCACAAGG GTCTCTGCTG 1200

ACTCCCCCGG CTCGGTCCAC AAGCTCTCCA CTTGCCCCTT TTAGGAAGTC CGGTCCCGCG 1260

GTTCGGGTAC CCCCTGCCCC TCCCATATTC TCCCGTCTAG CACCTTTGAT TTCTCCCAAA 1320

CCCGGCAGCC CGAGACTGTT GCAAACCGGC GCCACAGGGC GCAAAGGGGA TTTGTCTCTT 1380

CTGAAACCTG GCTGAGAAAT TGGGAACTCC GTGTGGGAGG CGTGGGGGTG GGACGGTGGG 1440

GTACAGACTG GCAGAGAGCA GGCAACCTCC CTCTCGCCCT AGCCCAGCTC TGGAACAGGC 1500

AGACACATCT CAGGGCTAAA CAGACGCCTC CCGCACGGGG CCCCACGGAA GCCTGAGCAG 1560

GCGGGGCAGG AGGGGCGGTA TCTGCTGCTT TGGCAGCAAA TTGGGGGACT CAGTCTGGGT 1620

GGAAGGTATC CAATCCAGAT AGCTGTGCAT ACATAATGCA TAATACATGA CTCCCCCCAA 1680

CAAATGCAAT GGGAGTTTAT TCATAACGCG CTCTCCAAGT ATACGTGGCA ATGCGTTGCT 1740

GGGTTATTTT AATCATTCTA GGCATCGTTT TCCTCCTTAT GCCTCTATCA TTCCTCCCTA 1800

TCTACACTAA CATCCCACGC TCTGAACGCG CGCCCATTAA TACCCTTCTT TCCTCCACTC 1860

TCCCTGGGAC TCTTGATCAA AGCGCGGCCC TTTCCCCAGC CTTAGCGAGG CGCCCTGCAG 1920

CCTGGTACGC GCGTGGCGTG GCGGTGGGCG CGCAGTGCGT TCTCTGTGTG GAGGGCAGCT 1980

GTTCCGCCTG CGATGATTTA TACTCACAGG ACAAGGATGC GGTTTGTCAA ACAGTACTGC 2040

TACGGAGGAG CAGCAGAGAA AGGGAGAGGG TTTGAGAGGG AGCAAAAGAA AATGGTAGGC 2100

GCGCGTAGTT AATTCATGCG GCTCTCTTAC TCTGTTTACA TCCTAGAGCT AGAGTGCTCG 2160

GCTGCCCGGC TGAGTCTCCT CCCCACCTTC CCCACCCTCC CCACCCTCCC CATAAGCGCC 2220

CCTCCCGGGT TCCCAAAGCA GAGGGCGTGG GGGAAAAGAA AAAAGATCCT CTCTCGCTAA 2280

TCTCCGCCCA CCGGCCCTTT ATAATGCGAG GGTCTGGACG GCTGAGGACC CCCGAGCTGT 2340

GCTGCTCGCG GCCGCCACCG CCGGGCCCCG GCCGTCCCTG GCTCCCCTCC TGCCTCGAGA 2400

AGGGCAGGGC TTCTCAGAGG CTTGGCGGGA AAAAGAACGG AGGGAGGGAT CGCGCTGAGT 2460

ATAAAAGCCG GTTTTCGGGG CTTTATCTAA CTCGCTGTAG TAATTCCAGC GAGAGGCAGA 2520

GGGAGCGAGC GGGCGGCCGG CTAGGGTGGA AGAGCCGGGC GAGCAGAGCT GCGCTGCGGG 2580

CGTCCTGGGA AGGGAGATCC GGAGCGAATA GGGGGCTTCG CCTCTGGCCC AGCCCTCCCG 2640

CTGATCCCCC AGCCAGCGGT CCGCAACCCT TGCCGCATCC ACGAAACTTT GCCCATAGCA 2700

GCGGGCGGGC ACTTTGCACT GGAACTTACA ACACCCGAGC AAGGACGCGA CTCTCCCGAC 2760

GCGGGGAGGC TATTCTGCCC ATTTGGGGAC ACTTCCCCGC CGCTGCCAGG ACCCGCTTCT 2820

CTGAAAGGCT CTCCTTGCAG CTGCTTAGAC GCTGGATTTT TTTCGGGTAG TGGAAAACCA 2880

GGTAAGCACC GAAGTCCACT TGCCTTTTAA TTTATTTTTT TATCACTTTA ATGCTGAGAT 2940

GAGTCGAATG CCTAAATAGG GTGTCTTTTC TCCCATTCCT GCGCTATTGA CACTTTTCTC 3000

AGAGTAGTTA TGGTAACTGG GGCTGGGGTG GGGGGTAATC CAGAACTGGA TCGGGGTAAA 3060

GTGACTTGTC AAGATGGGAG AGGAGAAGGC AGAGGGAAAA CGGGAATGGT TTTTAAGACT 3120

ACCCTTTCGA GATTTCTGCC TTATGAATAT ATTCACGCTG ACTCCCGGCC GGTCGGACAT 3180

TCCTGCTTTA TTGTGTTAAT TGCTCTCTGG GTTTTGGGGG GCTGGGGGTT GCTTTGCGGT 3240

GGGCAGAAAG CCCCTTGCAT CCTGAGCTCC TTGGAGTAGG GACCGCATAT CGCCTGTGTG 3300

AGCCAGATCG CTCCGCAGCC GCTGACTTGT CCCCGTCTCC GGGAGGGCAT TTAAATTTCG 3360

GCTCACCGCA TTTCTGACAG CCGGAGACGG ACACTGCGGC GCGTCCCGCC CGCCTGTCCC 3420

CGCGGCGATT CCAACCCGCC CTGATCCTTT TAAGAAGTTG GCATTTGGCT TTTTAAAAAG 3480

CAATAATACA ATTTAAAACC TGGGTCTCTA GAGGTGTTAG GACGTGGTGT TGGGTAGGCG 3540

CAGGCAGGGG AAAAGGGAGG CGAGGATGTG TCCGATTCTC CTGGAATCGT TGACTTGGAA 3600

AAACCAGGGC GAATCTCCGC ACCCAGCCCT GACTCCCCTG CCGCGGCCGC CCTCGGGTGT 3660

CCTCGCGCCC GAGATGCGGA GGAACTGCGA GGAGCGGGGC TCTGGGCGGT TCCAGAACAG 3720

CTGCTACCCT TGGTGGGGTG GCTCCGGGGG AGGTATCGCA GCGGGGTCTC TGGCGCAGTT 3780

GCATCTCCGT ATTGAGTGCG AAGGGAGGTG CCCCTATTAT TATTTGACAC CCCCCTTGTA 3840

TTTATGGAGG GGTGTTAAAG CCCGCGGCTG AGCTCGCCAC TCCAGCCGGC GAGAGAAAGA 3900

AGAAAAGCTG GCAAAAGGAG TGTTGGACGG GGGCGGTACT GGGGGTGGGG ACGGGGGCGG 3960

TGGAGAGGGA AGGTTGGGAG GGGCTGCGGT GCCGGCGGGG GTAGGAGAGC GGCTAGGGCG 4020

CGAGTGGGAA CAGCCGCAGC GGAGGGGCCC CGGCGCGGAG CGGGGTTCAC GCAGCCGCTA 4080

GCGCCCAGGC GCCTCTCGCC TTCTCCTTCA GGTGGCGCAA AACTTTGTGC CTTGGATTTT 4140

GGCAAATTGT TTTCCTCACC GCCACCTCCC GCGGCTTCTT AAGGGCGCCA GGGCCGATTT 4200

CGATTCCTCT GCCGCTGCGG GGCCGACTCC CGGGCTTTGC GCTCCGGGCT CCCGGGGGAG 4260

CGGGGGCTCG GCGGGCACCA AGCCGCTGGT TCACTAAGTG CGTCTCCGAG ATAGCAGGGG 4320

ACTGTCCAAA GGGGGTGAAA GGGTGCTCCC TTTATTCCCC CACCAAGACC ACCCAGCCGC 4380

TTTAGGGGAT AGCTCTGCAA GGGGAGAGGT TCGGGACTGT GGCGCGCACT GCGCGCTGCG 4440

CCAGGTTTCC GCACCAAGAC CCCTTTAACT CAAGACTGCC TCCCGCTTTG TGTGCCCCGC 4500

TCCAGCAGCC TCCCGCGACG ATGCCCCTCA ACGTTAGCTT CACCAACAGG AACTATGACC 4560

TCGACTACGA CTCGGTGCAG CCGTATTTCT ACTGCGACGA GGAGGAGAAC TTCTACCAGC 4620

AGCAGCAGCA GAGCGAGCTG CAGCCCCCGG CGCCCAGCGA GGATATCTGG AAGAAATTCG 4680

AGCTGCTGCC CACCCCGCCC CTGTCCCCTA GCCGCCGCTC CGGGCTCTGC TCGCCCTCCT 4740

ACGTTGCGGT CACACCCTTC TCCCTTCGGG GAGACAACGA CGGCGGTGGC GGGAGCTTCT 4800

CCACGGCCGA CCAGCTGGAG ATGGTGACCG AGCTGCTGGG AGGAGACATG GTGAACCAGA 4860

GTTTCATCTG CGACCCGGAC GACGAGACCT TCATCAAAAA CATCATCATC CAGGACTGTA 4920

TGTGGAGCGG CTTCTCGGCC GCCGCCAAGC TCGTCTCAGA GAAGCTGGCC TCCTACCAGG 4980

CTGCGCGCAA AGACAGCGGC AGCCCGAACC CCGCCCGCGG CCACAGCGTC TGCTCCACCT 5040

CCAGCTTGTA CCTGCAGGAT CTGAGCGCCG CCGCCTCAGA GTGCATCGAC CCCTCGGTGG 5100

TCTTCCCCTA CCCTCTCAAC GACAGCAGCT CGCCCAAGTC CTGCGCCTCG CAAGACTCCA 5160

GCGCCTTCTC TCCGTCCTCG GATTCTCTGC TCTCCTCGAC GGAGTCCTCC CCGCAGGGCA 5220

GCCCCGAGCC CCTGGTGCTC CATGAGGAGA CACCGCCCAC CACCAGCAGC GACTCTGGTA 5280

AGCGAAGCCC GCCCAGGCCT GTCAAAAGTG GGCGGCTGGA TACCTTTCCC ATTTTCATTG 5340

GCAGCTTATT TAACGGGCCA CTCTTATTAG GAAGGAGAGA TAGCAGATCT GGAGAGATTT 5400

GGGAGCTCAT CACCTCTGAA ACCTTGGGCT TTAGCGTTTC CTCCCATCCC TTCCCCTTAG 5460

ACTGCCCATG TTTGCAGCCC CCCTCCCCGT TTGTCTCCCA CCCCTCAGGA ATTTCATTTA 5520

GGTTTTTAAA CCTTCTGGCT TATCTTACAA CTCAATCCAC TTCTTCTTAC CTCCCGTTAA 5580

CATTTTAATT GCCCTGGGGC GGGGTGGCAG GGAGTGTATG AATGAGGATA AGAGAGGATT 5640

GATCTCTGAG AGTGAATGAA TTGCTTCCCT CTTAACTTCC GAGAAGTGGT GGGATTTAAT 5700

GAACTATCTA CAAAAATGAG GGGCTGTGTT TAGAGGCTAG GCAGGGCCTG CCTGAGTGCG 5760

GGAGCCAGTG AACTGCCTCA AGAGTGGGTG GGCTGAGGAG CTGGGATCTT CTCAGCCTAT 5820

TTTGAACACT GAAAAGCAAA TCCTTGCCAA AGTTGGACTT TTTTTTTTCT TTTATTCCTT 5880

CCCCCGCCCT CTTGGACTTT TGGCAAAACT GCAATTTTTT TTTTTTTATT TTTCATTTCC 5940

AGTAAAATAG GGAGTTGCTA AAGTCATACC AAGCAATTTG CAGCTATCAT TTGCAACACC 6000

TGAAGTGTTC TTGGTAAAGT CCCTCAAAAA TAGGAGGTGC TTGGGAATGT GCTTTGCTTT 6060

GGGTGTGTCC AAAGCCTCAT TAAGTCTTAG GTAAGAATTG GCATCAATGT CCTATCCTGG 6120

GAAGTTGCAC TTTTCTTGTC CATGCCATAA CCCAGCTGTC TTTCCCTTTA TGAGACTCTT 6180

ACCTTCATGG TGAGAGGAGT AAGGGTGGCT GGCTAGATTG GTTCTTTTTT TTTTTTTTTC 6240

CTTTTTTAAG ACGGAGTCTC ACTCTGTCAC TAGGCTGGAG TGCAGTGGCG CAATCAACCT 6300

CCAACCCCCT GGTTCAAGAG ATTCTCCTGC CTCAGCCTCC CAAGTAGCTG GGACTACAGG 6360

TGCACACCAC CATGCCAGGC TAATTTTTGT AATTTTAGTA GAGATGGGGT TTCATCGTGT 6420

TGGCCAGGAT GGTCTCTCCT GACCTCACGA TCCGCCCACC TCGGCCTCCC AAAGTGCTGG 6480

GATTACAGGT GTGAGCCAGG GCACCAGGCT TAGATGTGGC TCTTTGGGGA GATAATTTTG 6540

TCCAGAGACC TTTCTAACGT ATTCATGCCT TGTATTTGTA CAGCATTAAT CTGGTAATTG 6600

ATTATTTTAA TGTAACCTTG CTAAAGGAGT GATTTCTATT TCCTTTCTTA AAGAGGAGGA 6660

ACAAGAAGAT GAGGAAGAAA TCGATGTTGT TTCTGTGGAA AAGAGGCAGG CTCCTGGCAA 6720

AAGGTCAGAG TCTGGATCAC CTTCTGCTGG AGGCCACAGC AAACCTCCTC ACAGCCCACT 6780

GGTCCTCAAG AGGTGCCACG TCTCCACACA TCAGCACAAC TACGCAGCGC CTCCCTCCAC 6840

TCGGAAGGAC TATCCTGCTG CCAAGAGGGT CAAGTTGGAC AGTGTCAGAG TCCTGAGACA 6900

GATCAGCAAC AACCGAAAAT GCACCAGCCC CAGGTCCTCG GACACCGAGG AGAATGTCAA 6960

GAGGCGAACA CACAACGTCT TGGAGCGCCA GAGGAGGAAC GAGCTAAAAC GGAGCTTTTT 7020

TGCCCTGCGT GACCAGATCC CGGAGTTGGA AAACAATGAA AAGGCCCCCA AGGTAGTTAT 7080

CCTTAAAAAA GCCACAGCAT ACATCCTGTC CGTCCAAGCA GAGGAGCAAA AGCTCATTTC 7140

TGAAGAGGAC TTGTTGCGGA AACGACGAGA ACAGTTGAAA CACAAACTTG AACAGCTACG 7200

GAACTCTTGT GCGTAAGGAA AAGTAAGGAA AACGATTCCT TCTAACAGAA ATGTCCTGAG 7260

CAATCACCTA TGAACTTGTT TCAAATGCAT GATCAAATGC AACCTCACAA CCTTGGCTGA 7320

GTCTTGAGAC TGAAAGATTT AGCCATAATG TAAACTGCCT CAAATTGGAC TTTGGGCATA 7380

AAAGAACTTT TTTATGCTTA CCATCTTTTT TTTTTCTTTA ACAGATTTGT ATTTAAGAAT 7440

TGTTTTTAAA AAATTTTAAG ATTTACACAA TGTTTCTCTG TAAATATTGC CATTAAATGT 7500

AAATAACTTT AATAAAACGT TTATAGCAGT TACACAGAAT TTCAATCCTA GTATATAGTA 7560

CCTAGTATTA TAGGTACTAT AAACCCTAAT TTTTTTTATT TAAGTACATT TTGCTTTTTA 7620

AAGTTGATTT TTTTCTATTG TTTTTAGAAA AAATAAAATA ACTGGCAAAT ATATCATTGA 7680

GCCAAATCTT AAGTTGTGAA TGTTTTGTTT CGTTTCTTCC CCCTCCCAAC CACCACCATC 7740

CCTGTTTGTT TTCATCAATT GCCCCTTCAG AGGGCGGTCT TAAGAAAGGC AAGAGTTTTC 7800

CTCTGTTGAA ATGGGTCTGG GGGCCTTAAG GTCTTTAAGT TCTTGGAGGT TCTAAGATGC 7860

TTCCTGGAGA CTATGATAAC AGCCAGAGTT GACAGTTAGA AGGAATGGCA GAAGGCAGGT 7920

GAGAAGGTGA GAGGTAGGCA AAGGAGATAC AAGAGGTCAA AGGTAGCAGT TAAGTACACA 7980

AAGAGGCATA AGGACTGGGG AGTTGGGAGG AAGGTGAGGA AGAAACTCCT GTTACTTTAG 8040

TTAACCAGTG CCAGTCCCCT GCTCACTCCA AACCCAGGAA TT 8082

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4480 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGGGTTACAC GTCTTAACTC AGAGTTGCAA CAGGCTTGAA CAAGCCCAGG CACGCCCAGA 60

TACCTAGGGC CGAGTCACCG TTAAAACTAA CAGACCATAA AAGGAAAGGA ATACAGAACA 120

GACTAGGAGT ACCGGATCTG ACTCACAGGC CACCTGGCAG GAAGAGATAA GCCCCAGCCC 180

CCGACATTCA GGACGTCCCA GCCCGCACGT ACTCTTACCA TGTTACAACC TCATTCGAAT 240

ATGATTCAAA CCTGCCAATG TGTGTAGCTA TACCTTATCA CCTCATCTTG TGAAATAACC 300

AATCATATGT GAACATGTCT ATATGCTTCG TTTAAATCCA CCAATCCCCG TAACTATGCA 360

TCTGCTTCTG TACGCCCGCT TCTGCTTCCC CAAACCCTAT AAAAGCCCCA TGCTAGAGCT 420

GTTGGGCGCG CAAGTCCTCC GAAGAGACTG TGTGCCCGCA GGTACCTGTG TTTTCCAATA 480

AACCCTCTTG CTGATTGCAT CCGAGTGGCC TCGGCTCGGT CATTGGGCGC TTGGGGGTCT 540

CCTCCTGAGG GAAAGGTCCT CTCCGGAGGT CTTTTCATTT TGGGGGCTCG TCCGGGATCT 600

GGAGATCCTC CGCCCAGAGA TCACCGACCA CCCACCGGGA GGTAAGCCGG CCGGCATCTG 660

TCGTGTCTTG CCCTGTCTTG TCTTGTCTTG TCCTGTGCGC GTGTTCAGTT CGTCTCAGTT 720

TTGGACTCAG ATCTGGGTTT TGGTCGAAGG AGAAGGCCCA GGGCTTCGGT TTCTCAGGGT 780

TCAGGACCCT CAGCGCCTCC GTTTGGGCGG GTCAGAGAAG GAGCTGACGA GCTCGGACTT 840

CTCCCCCCGC AGCCCTGGAA GACGTTCCAA GGGTGTCTGG AGCCCGGTTC TTTGGGGCTC 900

AGCCCGTATC GGAGGGATAC GTGGTTTTGG TTGGAGGAGA GGGTCCAGGA CCCTCGGCAC 960

CTCCATCTGA CTCTTTGTTT TGGGTTTTAC GTCGAAGCCG CGCGGCGCGT CTGTCTGTTA 1020

TTTGTCTGAT CGTTGGATTT GTCTGTCTAA TCTGTGCCCT AATTTTCTTT GAAGCTACCA 1080

TGGGACAATC GCTAACAACC CCCTTGAGTC TCACTCTAGA CCATTGGAAG GACGTCCGAG 1140

ACCGAGCACG TGATCAGTCG GTCGAGATCA AGAAAGGTCC TCTCCGGAGG TCGGGGACAG 1200

TCGCGCCAGC AAGCGGTGGG GCAGGAGCTC CTGGTTTGGC AGCCCCTGTA GAAGCGATGA 1260

CAGAATACAA GCTTGTGGTG GTGGGCGCTA GAGGCGTGGG AAAGAGTGCC CTGACCATCC 1320

AGCTGATCCA GAACCATTTT GTGGACGAGT ATGATCCCAC TATAGAGGAC TCCTACCGGA 1380

AACAGGTAGT CATTGATGGG GAGACGTGTT TACTGGACAT CTTAGACACA GCAGGTCAAG 1440

AAGAGTATAG TGCCATGCGG GACCAGTACA TGCGCACAGG GGAGGGCTTC CTCTGTGTAT 1500

TTGCCATCAA CAACACCAAG TCCTTTGAAG ACATCCATCA GTACAGGGAG CAGATCAAGC 1560

GGGTGAAAGA TTCAGATGAT GTGCCAATGG TGCTGGTGGG CAACAAGTGT GACCTGGCCG 1620

CTCACACTGT TGAGTCTCGG CAGGCCCAGG ACCTTGCTCG CAGCTATGGC ATCCCCTACA 1680

TTGAAACATC AGCCAAGACC CGACCAGGTG TGGAGGATGC CTTCTACACA CTAGTACGTG 1740

AGATTCGGCA GCATAAACTG CGGAAACTGA ACCCGCCTGA TGAGAGTGGC CCTGGCTGCA 1800

TGAGCTGCAA GTGTGTGCTG TCCTGACACC AGGTTAAGGA CCTGATTTTC CGCCAGAAGC 1860

CGTACGGACA CCCTGACCAG GTGGCCTACA TTGTCACCTG GGAGAGCTTG GCATTTAGCC 1920

CTCCTCCTTG GGCAGAACCC TTTGTGGACC CGAATTGGCT TCCTGTTTCC CCTAAACCTG 1980

TTTCCCCGAG CCCACCTGAC CCTTTGGTTG CTTCTTCCTC TCTCTATCCT GCTCTAACTA 2040

AGGAAGAATC TCCCAAAGTC CCTCCCCCGA AACCTGTCCT CCCAGAGGAC CCAAATTCCC 2100

CCCTTATAGA TCTCCTGTTG GAAGAACCTC CTCCGTACCC TGTACCTACA GCCCCGCCAA 2160

GAGAAGAGGA AGTGGAGCCG CCTGCTAGAC CTCGACTCGA GGCGGCCCCT TCCCCTGTGG 2220

CTGGAAGACT TCGGGGACGA CGCGAGGTGG CGCCAGACTC CACCTCCCAG GCCTTTCCGC 2280

TTAGACAAGG GGCTGGCGGC CAGATACAAT ACTGGCCATT CTCAGCGGCC GACATATATA 2340

ACTGGAAACA ACACAACCCC CCCTTTTCTA AGGATCCGGT GGCTCTCACC AACCAGATAG 2400

AATCTGTCTT GCTTACCCAT CAGCCCACTT GGGATGATAT ACAGCAACTT TTACAGGCCC 2460

TCCTGACCTC TGAAGAGAAG CAGAGAGTGC TCTTAGAGGC CAGGAAACAT GTTTTGGGGG 2520

ACAATGGACG CCCCACCTTG CTCCCGAAAG AGATCGATGA TGCATTCCCA CTTACAAGAC 2580

CTGATTGGGA TTTCACCACG GCTAAAGGTA GGAGACACCT ACGCCTTTAT CGCCAGTTGC 2640

TCCTAGCGGG TCTCCGAGGG GCGGCACGAC GCCCCACCAA TTTGGCTCAG GTAAAACAAG 2700

TGGTACAAGA GGCTGCGGAG ACTCCCTCAG CCTTCCTAGA GAGACTTAAG GAAGCTTATC 2760

GCATGTATAC CCCTTATGAT CCAGATGATC CAGGACAAAT GACAAATGTC TCCATGTCCT 2820

TCATCTGGCA GGCAGCACCA GATATCAGGG CCAAGCTACA GAGAATAGAA AATTTACAAG 2880

GGTATACACT GCAGGATTTA CTTAAGGAGG CAGAAAGAAT TTATAACAAG AGAGAGACAC 2940

AAGAAGAAAA GAAAGATAAA ATACGTAGAG AAAAAGATGA GAGAGACCGA AAAAGAAACA 3000

GAGAGTTGAG TCGAATCTTG GCCGCCGTAG TTCAGGGTCA AGAGAAAAGG GGAGAGAGGG 3060

TGGGAGTTCG AAAGGGGCCA AAGCTAGATA AGGATCAATG TGCGTATTGC AAAGAAAGAG 3120

GACACTGGGC CAGAGATTGC CCTAAGAAAC CCAGCGGCTC CGAAGACCCC GCCCACAGAC 3180

CTCCCTCTTG GCCCTAGATA AAGATTAGGG AGGTCAGGGC CAGGAGCCCC CCCCTGAGCC 3240

CAGGATAACT CTTGAAGTTG GGGGGCAGCC AGTCACCTTT CTGGTGGACA CAGGAGCCCA 3300

GCACTCAGTC CTCACCCAGG CCCCTGGACA ACTCAGCGAC CGGACGGCCT GGGTACAAGG 3360

AGCCACTGGC AGCAAGAGAT ACCGTTGGAC TACAGATCGA CGGGTTCAGC TGGCTACTGG 3420

TAAGGTGACC CATTCCTTCT TACATGTTCC GGACTGCCCA TACCCTCTGC TGGGCCGTGA 3480

CTTGCTTACC AAATTAAAAG CTCAGATCCA TTTTGAAGAA GGAGGGACCC GAGTAACCGG 3540

GCCCCGCGGT ATTCCTCTTC AGATTTTAAC CCTTCAGTTA GAAGATGAAT ATAGATTATA 3600

TGAACCAGAA CAGGACAAGC CAAAATCTCC AGAAATAGAC TCTTGGGTCA CGAAATTCCC 3660

ACTGGCCTGG GCAGAGACTG GCGGGATGGG GTTGGCGCTC CAACAGCCTC CCCTAATTAT 3720

CCAGTTAAAG GCCACCGCGA CTCCTGTCTC CATTAAACAG TACCCCATGT CATGGGAAGC 3780

TTATCAGGGC ATAAAGCCAC ATATCAGGAG GCTCTTAGAC CAAGGCATCC TAGTCCCTTG 3840

CCGGTCACCC TGGAATACGC CTCTGCTACC TGTTAAGAAG CCCGGCACTG GAGACTATAG 3900

GCCAGTACAA GATTTGAGAG AGGTCAACAA AAGAGTAGAA GATATTCATC CAACTGTCCC 3960

AAACCCTTAT AACCTACTCA GCACCCTGCC TCCCACCCAT ACTTGGTATA CGGTCTTAGA 4020

TCTGAAGGAT GCTTTCTTCT GCCTCCGGCT GAGCCCAGAA AGCCAGCCCT TATTTGCTTT 4080

TGAGTGGAAA GACTCTGAAA TGGGGCTTTC GGGACAGTTG ACTTGGACAA GGTTACCACA 4140

GGGTTTCAAA AACAGCCCAA CGCTCTTTGA TGAGGCCTTA CACCGGGACT TGGCTGACTT 4200

TCGAGTCCAG CATCCCACTC TTATACTTCT TCAGTTTGTT GATGACCTTC TTCTAGGGGC 4260

CACTTCTGAG ACAGCATGCC ACCAGGGAAC AGAATCCCTC TTGCAGACTT TGGGGCGATT 4320

GGGCTATCGA GCTTCTGCCA GAAAGGCTCA AATTTGCCAG ACCCAGGTTA CTTATTTAGG 4380

CTATCAACTA AGGGATGGAC AGCGATGGCT GACTCCGGCT AGGAAACAGA CCGTGGCCAA 4440

CATCCCAGCC CCAAGAAATG GCCGACAGCT ACGGGAATTC 4480

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 565 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCTGAGTAGT GCGCGAGCAA AATTTAAGCT ACAACAAGGC AAGGCTTGGC CGACAATTGC 60

ATGAAGAATC TGCTTAGGGT TAGGCGTTTT GCGCTGCTTC GCGATGTACG GGCCAGATAT 120

ACGCGTATCT GAGGGGACTA GGGTGTGTTT AGGCGAAAAG CGGGGCTTCG GTTGTACGCG 180

GTTAGGAGTC CCCTCAGGAT ATAGTAGTTT CGCTTTTGCA TAGGGAAGGG GAAATGTAGT 240

CTTATGCAAT ACTCTTGTAG TCTTGCAACA TGCTTATGTA ACGATGAGTT AGCAACATGC 300

CTTACAAGGA GAGAAAAAGC ACCGTGCATG CCGATTGGTG GAAGTAAGGT GGTACGATCG 360

TGCCTTATTA GGAAGGCAAC AGACGGGTCT GACATGGATT GGACGAACCA CCGAATTCCG 420

CATTGCAGAG ATATTGTATT TAAGTGCCTA GCTCGATACA ATAAACGCCA TTTGACCATT 480

CACCACATTG GTGTGCACCT GGGTTGATGG CCGGACCGTT GATTCCCTGA CGACTACGAG 540

CACCTGCATG AAGCAGAAGG CTTCA 565

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1804 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGATCCTCAG GGGTAACACC TTTTGGAGGT GGGCATCTTC CTCATTCTCA GTGGTGCCAA 60

GTTCATATCC TGCTGGCTTA ACACGTGGTG TTACTATATT TGTGGCCTTA TATGATTATG 120

AAGCTAGAAC TACAGAAGAC CTTTCATTTA AGAAGGGTGA AAAATTTCAA ATAATTAACA 180

ATACAGAAGG AGACTGGTGG GAAGCAAGAT CAATCACTAC AGGAAAGAAT GGTTATATCC 240

TGAGCAGTTA TGTAGCGCCT GCAGATTCCA TTCAGGCAGA AGAATGGTAT TTTGGCAAAA 300

TGGGGAGAAA AGATGCTGAA AGATTACTTC TGAATCCTGG AAATTAATGA GGTATTTTCT 360

TAGGAAGAGA GAGTGAAATG GCTGGGTGCA GTGGCTCATG CCTGTAATCC CAGCACTTTG 420

GGAGGCCGAG TTGGGCGGAT CACCTGAGGT CAGGAGTTCG AGACTAGCCT GGCCAACATG 480

GTGAAACCCC ATCTCTACTA AAAAAAAAAG TACAAAATTA GCTGGACGTG GTGGTGAGTG 540

CCTGTAATCC CAGCTACTCA GGAGGCTGAG GCAGCAGAAT CACTTGAACC TGGGAGGCGG 600

AGGTTGCAGT GAGCTGAGAT CGCGCCACTG CACTCCAGCC TCGGCGACAA GAGCAAAAAC 660

TCCGTCTAAA AAACAAATAA GCAAACAGAA CAAAACAAAA CAAAAACGAG AGAGCGAAAC 720

TACTAAAGGT GCTTATTCCC TCTCTATTCG TGATTGGGAT GAGGTAAGGG GTGACAATGT 780

GAAACACCAC AAAATTAGGA AACTTGACAA TGGTAGATAC TATATCACAA CCAGAGAACA 840

ACTTGATACT CTGCAGAAAT TGGCAAAACA CTACACAGAA CATGCTGATG GTTTATGCCA 900

CAAGTTAACA ACTGTGTGTC CAACTGTGAA ACCTCAGATT CAAGGTCTAG CAAAAGATGC 960

TTGGGAAATC CCTTGATAAT CTTTGCGACT AGAGGTTAAA CTAGGACAAG GATGTTTTGG 1020

CAAAGTGTGG ATGGGAATAT GGAATGGAAC CACAAAAGTA GCAATCAAAA CACTAAAACC 1080

AGGTACAATG ATGCCAGAAG CTTTTCTTCA AGAAGCTCAG GTAATGAAAA AAATAAGACA 1140

TGGTAAACTT GTTCCACTAT ATGCTGTTGT TTCTGAAGAG CCAATTTACA TTGTCACTGA 1200

ATTGATGTCA AAAGGAAGCT TATTCAATTT CCTTAAGGAA GGAGATGGAA AGTATTTGAA 1260

GCTTCCACAA ATGGTTGATA TGCCTGCTCA GATTGCTGAT GGTATGGCAT ATATTAAAAG 1320

AATGAACTAT ATTCACCGAG ATCTCTGGGC TGCTAATATT CTTGTAGGAG AAAATCTTCT 1380

GTGCAAAATA GCAGATTTTG GTTTAGCAAG GTTAATTGAA GACAATGAAT ACACATCAAG 1440

ACAAGGTGCA GAATTTCCAA TCAAATGGAC AGCTCCTGAA GTTGCACTGT ATGGTGGGTT 1500

TACAATAAAG TCTGGTGTCT GCTCATTTGG AATTCTACAG ACAGAACTGG TAACAAAGGG 1560

CAGAGTGCCA TATCCAGGTA TGGTGAACCA TGAAATACTG GAACAGGTGG AGCGAGGATA 1620

CAGGATGCCT TGCCCTCAGG GCTGTCCAGA ATCCCTCCAT GAATTGATGA ATCTGTGTTG 1680

GAAGAAGGAC CCTGATGAAA GACCAACATT TGAATATGTT CAGTCCTTCT TGGGAGACTA 1740

CTTCACTGCT ACAGAGCCAT AGTACCAGCC AGGAGAAAAC TTCTAATTCA AGTAGCCTAT 1800

TTTA 1804

Claims

1. A cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which target proto-oncogene is associated with a cancer, which cellular immunogen comprises host cells which have been transfected with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene.

2. An immunogen according to claim 1 wherein the transgene comprises

wild-type or mutant retroviral oncogene DNA; or

wild-type or mutant proto-oncogene DNA of a species different from the host species.

3. An immunogen according to claim 2 wherein the transfected cells are non-dividing.

4. An immunogen according to claim 2 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.

5. An immunogen according to claim 4 wherein the mutant DNA is nontransforming.

6. An immunogen according to claim 5 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

7. A cellular immunogen according to claim 6 wherein the host cells have been transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

8. An immunogen according to claim 1 wherein the host cells have been transfected with a transgene cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-*erbB*-2, MDM-2, c-*myc*, c-*myb*, c-*ras*, c-*src* and c-*yes*.

9. An immunogen according to claim 1 wherein the cells comprise fibroblasts.

10. A method for preparing a cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which target proto-oncogene is associated with a cancer, the method comprising:

(a) excising cells from the host;

(b) transfecting the excised cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene.

11. A method according to claim 11 wherein the transgene comprises

wild-type or mutant retroviral oncogene DNA; or

wild-type or mutant proto-oncogene DNA of a species different from the host species.

12. A method according to claim 11 wherein the transfected cells are non-dividing.

13. A method according to claim 11 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.

14. A method according to claim 13 wherein the mutant DNA is nontransforming.

15. A method according to claim 14 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

16. A method according to claim 15 wherein the host cells are transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

17. A method according to claim 11 wherein the transgene is cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-*erbB*-2, MDM-2, c-*myc*, c-*myb*, c-*ras*, c-*src* and c-*yes*.

18. A method according to claim 1 wherein the excised cells comprise fibroblasts.

19. A method of vaccinating a host against disease associated with the overexpression of a target proto-oncogene comprising

(a) excising cells from the host;

(b) transfecting the excised cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of

the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene;

(c) returning the excised cells transfected with the transgene construct to the body of the host to obtain expression of the transgene in the host.

20. A method according to claim 19 wherein the transgene comprises

wild-type or mutant retroviral oncogene DNA; or

wild-type or mutant proto-oncogene DNA of a species different from the host species.

21. A method according to claim 20 wherein the transfected cells are rendered non-dividing prior to return to the body of the host.

22. A method according to claim 20 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.

23. A method according to claim 22 wherein the mutant DNA is nontransforming.

24. A method according to claim 23 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

25. A method according to claim 24 wherein the host cells are transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

26. A method according to claim 19 wherein the transgene is cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-*erbB*-2, MDM-2, c-*myc*, c-*myb*, c-*ras*, c-*src* and c-*yes*.

27. A method according to claim 19 wherein the excised host cells comprise fibroblasts.

28. A method of vaccinating a host against disease associated with the overexpression of a targeted proto-oncogene comprising

(a) excising cells from the host;

(b) transfecting the excised cells with at least one transgene construct comprising at least transgene and a strong promoter to drive the expression of the transgene in the transfected cells, wherein the transgene comprises

(1) wild-type or mutant cognate retroviral oncogene DNA; or

(2) wild-type or mutant cognate proto-oncogene DNA of a species different from the host species;

(c) returning the excised cells transfected with the transgene construct to the body of the host to obtain expression of the transgene in the host.

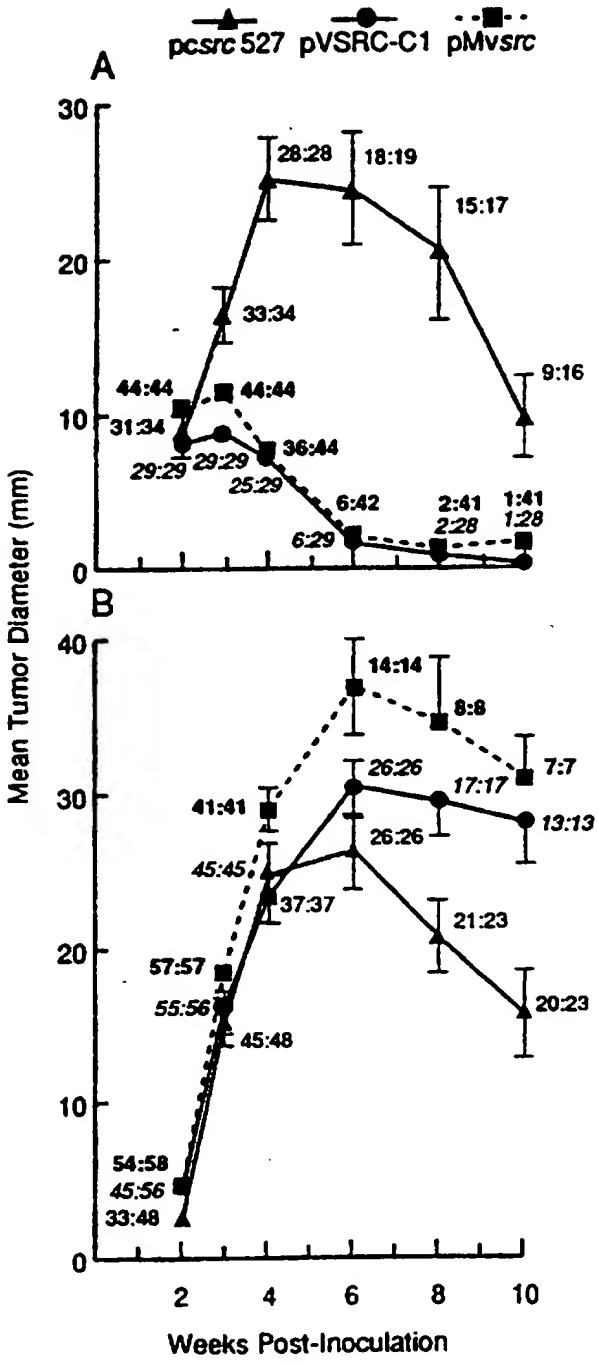
pcsrc 527
pVSRC-C1
pMvsrc
A
B
Mean Tumor Diameter (mm)
Weeks Post-Inoculation
28:28
18:19
15:17
33:34
9:16
44:44
44:44
31:34
29:29
29:29
36:44
25:29
6:42
2:41
1:41
2:28
1:28
6:29
14:14
8:8
7:7
26:26
17:17
41:41
13:13
26:26
45:45
37:37
21:23
57:57
20:23
55:56
45:48
54:58
45:56
33:48

FIG. 1

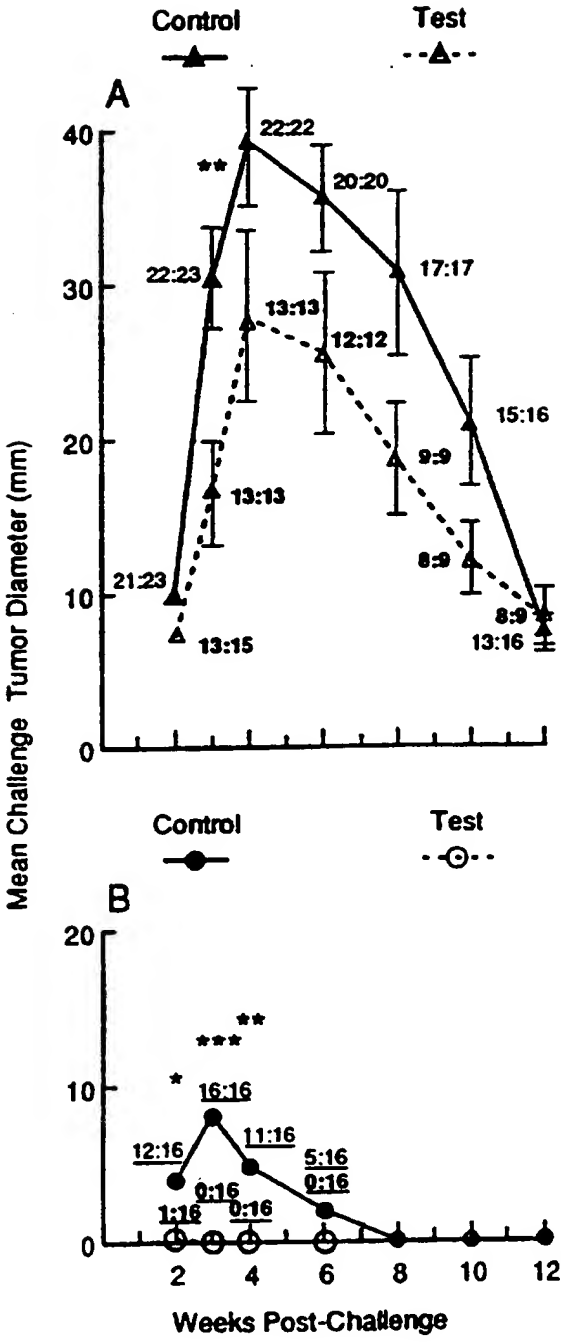
Control
Test
A
40
30
20
10
0
22:22
20:20
17:17
15:16
22:23
13:13
12:12
9:9
13:13
8:9
21:23
8:9
13:15
13:16
Mean Challenge Tumor Diameter (mm)
Control
Test
B
20
10
0
16:16
11:16
12:16
5:16
0:16
0:16
1:16
0:16
2
4
6
8
10
12
Weeks Post-Challenge

FIG. 2

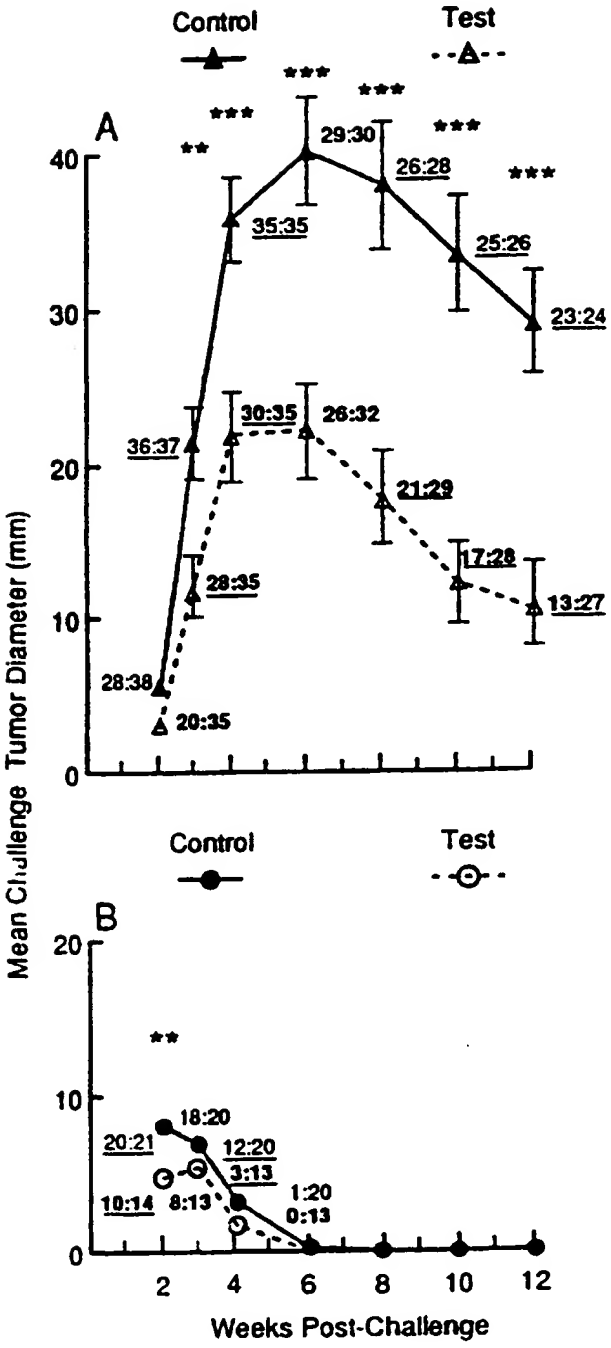
Control
Test
A
Mean Challenge Tumor Diameter (mm)
40
30
20
10
0
***
**
29:30
26:28
25:26
23:24
35:35
36:37
30:35
26:32
21:29
17:28
13:27
28:35
28:38
20:35
Control
Test
B
20
10
0
**
18:20
20:21
12:20
3:13
10:14
8:13
1:20
0:13
2
4
6
8
10
12
Weeks Post-Challenge

FIG. 3

INTERNATIONAL SEARCH REPORT

Inte. .tional application No.
PCT/US97/00582

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01K 63/00; A61K 39/00, 39/38, 48/00; C12N 5/00, 15/00

US CL : 424/93.21, 184.1; 435/172.1, 240.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21, 184.1; 435/172.1, 240.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPAT, MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS
Search terms: oncogene/transfection/vaccine/proto-oncogene

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MALONE et al. Cationic liposome-mediated RNA transfection. Proc. Natl. Acad. Sci. USA. August 1989, Vol. 86, pages 6077-6081, see entire document	1-18
Y,E	US 5,593,972 (WEINER et al.) 14 January 1997, see entire document.	1-28
Y	FENDLY et al. The extracellular domain of HER2/neu is a potential immunogen for active specific immunotherapy of breast cancer. J. Biol. Response Mod. October 1990, Vol. 9, No. 5, pages 449-455, see entire document.	1-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 MARCH 1997	Date of mailing of the international search report 12 MAY 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RAY F. EBERT Telephone No. (703) 3058-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Inter ..ational application No.
PCT/US97/00582

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FELGNER et al. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA. November 1987, Vol. 84, pages 7413-7417, see entire document.	1-28
Y	FENTON et al. Cytotoxic T-cell response and In Vivo protection against tumor cells harboring activated ras proto-oncogenes. J. Natl. Cancer Inst. 18 August 1993, Vol. 85, No. 16, pages 1294-1302, see entire document.	1-28
Y	MCCABE et al. Minimal determinant expressed by a recombinant vaccinia virus elicits therapeutic antitumor cytolytic T lymphocyte responses. Cancer Res. 15 April 1995, Vol. 55, pages 1741-1747, see entire document.	1-28
Y	TEMIN, H.M. Overview of biological effects of addition of DNA molecules to cells. J. Med. Virol. May 1990, Vol. 31, pages 13-17, see entire document.	1-28
Y	CONRY et al. Characterization of a messenger RNA polynucleotide vaccine vector. Cancer Res. 01 April 1995, Vol. 55, pages 1397-1400, see entire document.	1-28